"Pharmacological screening of some herbal extracts & fractions upon asthma, arthritis along with Alzheimer's & neurodegeneration related dementia in animal models with molecular target-based drug discovery approach"

Thesis submitted by

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i) Partha Palit, Dhrubojyoti Mukherjee, Subhash C. Mandal. "Reconstituted mother tinctures of *Gelsemium sempervirens* L. improve memory and cognitive impairment in mice scopolamine-induced dementia model". *Journal of Ethnopharmacology*, 159; 274-284, (2015).

ii) Partha Palit, Dhrubojyoti Mukherjee, Poulami Mahanta, M Shadab, Nahid Ali, Shubhadeep Roychoudhury, MD Asad, Subhash C. Mandal. "Attenuation of nociceptive pain and inflammatory disorders by total steroid and terpenoid fraction of *Euphorbia tirucalli* Linn root in experimental in vitro and in vivo model". *Inflammopharmacology*, 26; 235-250, (2018).

iii) Dhrubojyoti Mukherjee, Partha Palit, Shubhadeep Roychoudhury, Sukalyan K. Kundu, Subhash C. Mandal. "Role of Stress in Diseases and Its Remedial Approach by Herbal and Natural Products in Stress-Related Disease Management: Experimental Studies and Clinical Reports". *Natural Products and Drug Discovery*, Chapter 14, Elsevier, 375-410, (2018).

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ii) Dhrubojyoti Mukherjee, Partha Palit, Subhash C. Mandal. "Reconstituted mother tinctures of *Gelsemium sempervirens L.* improve memory and cognitive impairment in mice scopolamine-induced dementia model", dated 27th – 29th April 2022, at Virtual International Conference on Emerging Trends in Biotechnology and Sustainable Chemistry ETBSC-2022, organized by the Department of Biotechnology and Chemistry Faculty of Sciences, Baba Farid College, Bathinda, India.

iii) Dhrubojyoti Mukherjee, Partha Palit, Subhash C. Mandal. "Reconstituted mother tinctures of Gelsemium sempervirens L. improve memory and cognitive impairment in mice scopolamine-induced dementia model", dated 7th May 2022, at National Conference on Intellectual Property Rights of Pharmaceuticals, sponsored by AICTE, scheme for promoting interests creativity and ethics among students (SPICES), organized by Pranveer Singh Institute of Technology, Kanpur, India. *Awarded 2nd prize in the oral presentation*.

PROFORMA – 1

"Statement of Originality"

I Dhrubojyoti Mukherjee registered on 23/04/2015 do hereby declare that this thesis entitled " Pharmacological screening of some herbal extracts & fractions upon asthma, arthritis along with Alzheimer's & neurodegeneration related dementia in animal models with molecular target-based drug discovery approach" contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies.

All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work.

I also declare that I have checked this thesis as per the "Policy on Anti Plagiarism, Jadavpur University, 2019", and the level of similarity as checked by iThenticate software is____6_____%.

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<center>PROFORMA - 2</center>

<center>## CERTIFICATE FROM THE SUPERVISORS</center>

This is to certify that the thesis entitled **"Pharmacological screening of some herbal extracts & fractions upon asthma, arthritis along with Alzheimer's & neurodegeneration related dementia in animal models with molecular target-based drug discovery approach"** submitted by Shri Dhrubojyoti Mukherjee, who got his name registered on 23/04/2015 for the award of Ph.D. (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of Prof. (Dr.) Subhash C. Mandal (Dept of Pharm Tech, Jadavpur University), Dr. Shubhadeep Roychoudhury (Asst. Prof., Dept of Lifescience & Bioinformatics, Assam University, Silchar) and Dr. Partha Palit (Asst. Prof., Dept of Pharmaceutical sciences, Assam University, Silchar) and that neither his thesis nor any part of the thesis has been submitted for any degree/ diploma or any other academic award anywhere before.

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PREFACE

The use of herbal medicine becoming popular due to toxicity and side effects of allopathic medicines. Medicinal plants play an important role in the development of potent therapeutic agents. Over the past decade, herbal and ayurvedic drugs from plant kingdom has become a subject of world importance, with both medicinal and economic implications. A regular and widespread use of herbs throughout the world has increased serious concerns over their quality, safety and efficacy. Thus, a proper scientific evidence or assessment has become the criteria for acceptance of herbal health claims. Therefore, number of researchers are trying to develop various antiasthmatic, anti-inflammatory, antialzhemers drugs scientifically from herbal sources.

However, the scientific literature supporting the efficacy of herbal therapies is incomplete. There are few well-controlled studies that support the efficacy of herbal remedies in the treatment and clinical improvement of patients with asthma, arthritis and Alzheimer's disease. Available scientific evidence has not yet confirmed the validity of their popular role in the treatment of these diseases. Worldwide researchers are now focusing on drug discovery from herbal sources through battery of *in vitro* and *in vivo* assays in respective therapeutic models. Many pharmaceutical firms are currently focusing on the development of plant-derived medications, given that the production of synthetic compounds for therapeutic use is, by and large, a random process that may result in fortuitous discovery.

In this proposal an attempt was done to find out novel medicinal plants having anti-arthritic, antiasthmatic, anti-inflammatory and anti-Alzheimer potential involvement of their chemical constituents and pharmacological profile. Respective plant fractions were assessed through various *in vitro* and *in vivo* animal models of arthritis, asthma and dementia for evaluating the therapeutic prospect.

Abbreviations

Sr. No	Abbreviations	Full form
1	IgE	Immunoglobulin E
2	WHO	World Health Organisation
3	COPD	Chronic obstructive pulmonary disease
4	OTC	Over the counter
5	OA	Osteoarthritis
6	NSAIDs	Nonsteroidal anti-inflammatory drugs
7	SLE	Systemic lupus erythematosus
8	RA	Rheumatoid arthritis
9	CVD	Cardiovascular disease
10	GCs	Glucocorticoids
11	DMARDs	Disease-modifying anti-rheumatic drugs
12	FCA	Freund's complete adjuvant
13	AD	Alzheimer's disease
14	APP	Amyloid precursor protein
15	NPC	Neural progenitor cells
16	ChEIs	Cholinesterase inhibitors
17	OECD	Organization for Economic Co-operation and Development
18	WFI	Water for injection
19	EPM	Elevated plus maze
20	TL	Transfer latency
21	SDL	Step-down latency
22	AChE	Acetylcholinesterase
23	LPS	Lipo-poly saccharide
24	DPPH	2,2-diphenyl-1-picrylhydrazyl
25	HRBC	Human red blood cell
26	LOX	Lipoxygenase
27	PBMCs	Peripheral blood mononuclear cells
28	TNF	Tumor necrosis factor
29	STF-HAETR	Steroid and terpenoid rich fractions of E. tirucalli root hydro-alcoholic extract
30	iNOS	Inducible nitric oxide synthase
31	PVDF	Polyvinylidene fluoride
32	COX	cyclooxygenase
33	LOX	Lipoxygenase
34	BSA	Bovine serum albumin
35	PBMC	Peripheral blood mononuclear cells
36	FGWE	Fenugreek water extract
37	BGWE	Bottle gourd water extract

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I. Introduction

1.1 Introduction:

According to research, global prescription medicine spending reached US$ 643 billion in yearly sales in 2006 (Pan et al., 2013). Despite the lengthy development process, only one or two, out of every ten thousand of these chemical compounds be clinically effective and safe enough for regulatory approval in clinical process. In reality, nearly half of all medication candidates fail in clinical trials towards the end. Many pharmaceutical firms are currently focusing on the development of plant-derived medications, given that the production of synthetic compounds for therapeutic use is, by and large, a random process that may result in fortuitous discovery. Inflammation, often known as the general immunological response, has both positive and negative consequences. While the inflammatory response is mostly used as a protective response to remove and repair wounded tissues or degrading stimuli, mismanagement of the response can lead to chronic inflammation. Chronic, low-grade inflammatory illnesses like arthritis and asthma, which afflict 0.5-2% of the human population, can fester for a long time and negatively contribute to the development of diseases linked with age, such as arthritis and systemic autoimmune disease.

1.2 Basic consideration of asthma:

Asthma is a chronic inflammatory condition characterised by airway constriction and changing numbers of lymphocytes, eosinophils, cytokines, mast cells, also some inflammatory cellular products. Normally it is identified that asthmatic patient is seen with increased amounts of particular immunoglobulin E (IgE), seen binding with mast

cells and different inflammatory cell receptors. The interacting IgE antibody with the antigens triggers a cascade of inflammatory cellular responses, that includes production of various mediators like prostaglandins, leukotrienes, and histamines that causes bronchoconstriction and airways smooth muscle contraction (Holgate et al., 2008; Del Donno et al., 2000). Despite improvements in the general health of the population, the rate of asthma is rising (Ring et al., 2001). Asthma causes high morbidity and has a significant economic burden (Spector et al., 1997). They're on the rise as a result of pollution and people's growing proclivity for creating excessive IgE. (Passali et al., 1999). According to WHO estimates, 300 million people have been suffering from asthma, with 255,000 people dying from the disease in 2005. By 2025, it is anticipated that an extra 100 million people would have asthma. Inhaled corticosteroids and β2 agonist bronchodilators can help to manage it briefly. REfs However, an allopathic medication that can permanently cure it is still a long way off (Opina et al., 2017). The function of immunoglobulins, lymphocytes, mast cells, and different autacoids in etiopathogenesis of allergy related diseases has been extensively studied during the last several decades. Despite the large body of knowledge on the issue, the management of allergic disorders remains insufficient. Due to limited effectiveness, adverse reactions, toxicity, and side effects associated with synthetic allopathic medications, the existing treatment choices for upper and lower respiratory tract allergic disorders and bronchial asthma have significant limits (Salib et al., 2003). Asthma prevalence, morbidity, death, and economic burden have all grown globally since the 1970s, particularly in children (Szelenyi et al., 2002).

1.3 Pathophysiology and etiology of asthma:

Asthma has no one cause, and it is thought to be influenced by a variety of environmental and genetic variables. Premature delivery, low birth weight, and cigarette smoke exposure are among them (especially if the mother smokes in pregnancy). Prepubescent boys have higher chances of developing asthma, whereas girls tend to develop it during adolescence. Exposure to plastics, agricultural chemicals and volatile solvents may also responsible for the development of asthma. Asthma is more common in developed countries. Changes in housing, air pollution levels, and a more sanitary lifestyle (cutting early allergy exposure) is all seen to be connected with an increasing incidence of asthma in recent decades (Kaufman., 2011).

Asthma is typically triggered by an allergen that tends to produce an allergic response which is mediated by an immunoglobulin (IgE). The production of IgE is seen in reaction to the allergens including animal dander and pollens. During the initial exposure it is seen that allergen-specific IgE antibodies causes sensitisation by binding to the mast cells' surface (Barnes., 2011). The allergen when attached to allergen-specific IgE antibodies on the mast cells surface; histamines, prostaglandins and leukotrienes very well known as the inflammatory mediators are released as a consequence of mast cell degranulation **(Figure 1.1)**.
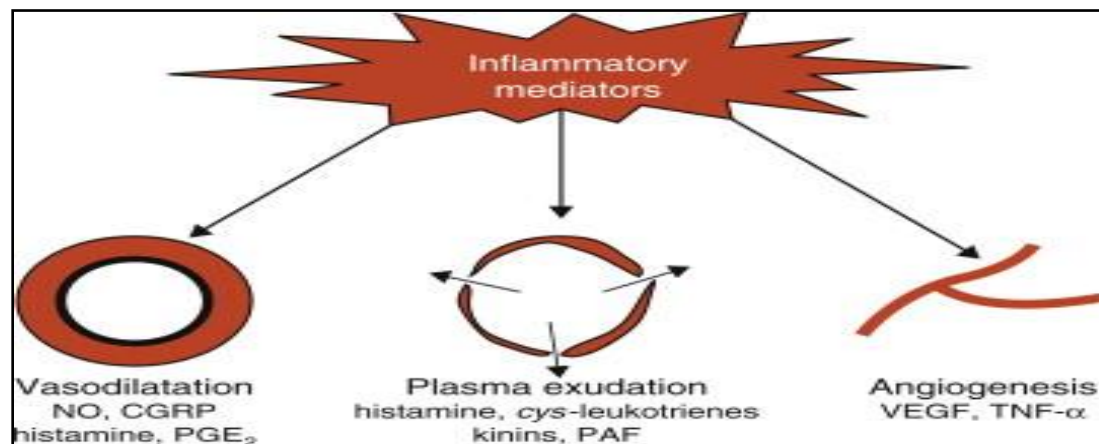
Figure 1.1: Release of inflammatory mediators in response to allergens (Peter et al.,

2009)

The inflammatory mediators then cause bronchospasm, that leads to an asthmatic episode. The T-helper cells, eosinophils, and mast cells travel through the airway if the particular attack is left untreated. The airway gets clogged due to excess production of mucus by the goblet cells. Alongside with hyperresponsiveness and elevated airways tone it reasons the airways to shrink, worsening the symptoms (Dournes et al., 2012). Some evidence suggests that poorly treated asthma for a longer duration leads to remodelling of the airways. Bronchial smooth muscle hypertrophy, development of new artery, and interstitial collagen deposition all are resulted due to chronic inflammation, ensuing in persistent blockage of airflow similar to chronic obstructive pulmonary disease (COPD) individuals. **(Figure 1.2)**.
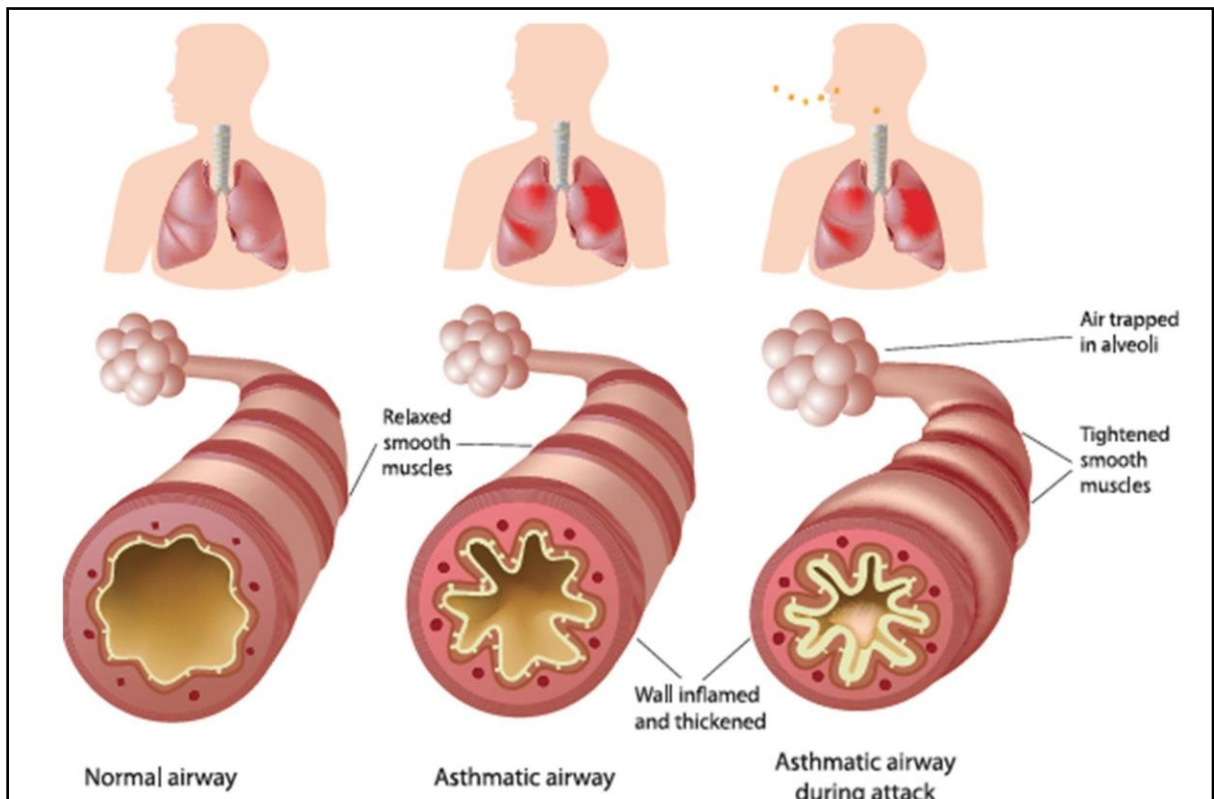
Figure 1.2: Comparative bronchial airway showing shrinkage

(https://www.weilab.com)

1.4 Current treatment of asthma and drawbacks

Current national and international management of asthma recommendations propose a progressive strategy, with therapy increasing until asthma is cured. Anti-inflammatory medications and bronchodilators are now available that show good effects, and most patients may obtain good asthma control (Choby et al., 2015). However, a large percentage is still seen to have severe chronic asthma which is challenging to control and maybe require different treatments.

a. β2 agonists:

Salbutamol and terbutaline, inhaled short-acting β2-agonists, are efficient bronchodilators and are recommended to all symptomatic asthma patients. It is used for acute severe asthma and in avoiding exercise-induced asthma symptoms when taken prior to commencement of the exercise. But the use of Salbutamol is restricted or avoided due to restlessness, nervousness, palpitation, muscle tremors (dose-related), ankle edema and throat irritation. Similarly, Terbutaline treatment is also struggling from various drawbacks including tremors, palpitations, muscle cramps, and dry mouth. Furthermore, they do not have any anti-inflammatory properties. To alleviate the symptoms, this therapy is used as needed. Although studies have indicated that the therapy has limited advantages, it may also be hazardous (Choucair-Jaafar et al., 2011).

b. Inhaled corticosteroid:

Inhaled corticosteroids are now indicated to all individuals with chronic asthma that requires short-acting β2-agonists more than thrice per day. They have anti-inflammatory properties via a variety of pathways, that includes glucocorticoid receptor activation. Systemic adverse effects are normally not noticeable in low dosages, daily 800 µg of beclomethasone dipropionate or 500 µg of fluticasone or budesonide, but they do become a concern at higher levels. Dysphonia and oral candidiasis are both typical side effects of inhaled corticosteroids (Kennedy et al., 2000). Skin atrophy and bruising, as well as a reduction in bone mineral density, are all systemic adverse effects (Mak et al., 1992).

c. Cromones:

Inhalation of the cromones, nedocromil sodium and sodium cromoglycate has been utilised as a controller therapy for moderate persistent asthma. They are thought to inhibit inflammatory cells and reduce IgE-mediated inflammatory responses. Sodium cromoglycate showed proven improvement of symptoms, airway responsiveness, and lung function, while nedocromil sodium have demonstrated to lower symptoms and aggravation of frequency (Diaz et al., 1984). They show low efficacy than low-dose inhaled corticosteroids in the long run, and the long-lasting consequences on inflammation of airways is uncertain. The introduction of modest dosages of inhaled steroids for majority of patients having chronic asthma has essentially replaced the use of these drugs in adults (Szefler et al., 1998).

d. Leukotriene antagonists

Zafirlukast and montelukast both are cysteinyl leukotriene receptor antagonists that may significantly reduce exercise-induced bronchoconstriction along with inhaled allergens early and late responses. Clinical studies have indicated improved lung functioning, a reduced requirement for rescue bronchodilators, and few indications of a reduced eosinophilic airways inflammation when added to as-needed $\beta 2$-agonists. In one of the studies, it was observed that patients who were given leukotriene receptor antagonists had both psychiatric and non-psychiatric adverse effects. Psychiatric issues were the most frequently noticed that restricted the use of leukotriene receptor antagonists (Erdem et al., 2015).

e. Theophylline:

For many years, theophylline has been used as a bronchodilator in quite large dosages but owing to side effects, it is frequently reserved for individuals with severe asthma. Gastrointestinal discomfort is the most prevalent side effect, but arrhythmia and tachycardia can sometimes happen, and with high-dosing therapy the serum concentration tests are usually recommended (Pollard et al., 1997).

f. Oral corticosteroids and corticosteroid sparing agents:

Another unit of individuals has severe chronic asthma that is hardly manageable even with the said interventions. Treating with oral corticosteroids, generally with daily prednisolone, that might be necessary for these conditions to reduce symptom and prevents serious asthma aggravations. Although oral corticosteroids are certainly an important element of the therapy of acute exacerbations, they need to be given carefully before being used long-term due to the high risk of major side effects. When they're needed, the lowest dose that keeps asthma under control is provided (Allen et al., 1994). Methotrexate, gold, and cyclosporin are examples of corticosteroid-sparing drugs. While there are few evidence showing these drugs can reduce usage of steroid in asthma, they respectively have their set of safety issues, with limited use to specialised centres. Because of the risky side effects with long-term usage of oral corticosteroids along with absence of acceptable substitutes, the response to therapy needs to be closely monitored (Lock et al., 1996).

g. Anticholinergics:

Dryness in the mouth, swallowing difficulty, photophobia, palpitation, hallucinations, cardiovascular system collapse, convulsions, and coma are some of the side effects of the anticholinergics used in the treatment of asthma (Taur et al., 2011).

1.5 Role of herbal medicines in the treatment of asthma:

Despite the vast amount of knowledge on the issue, the management of allergic disorders remains unsatisfactory. Due to limited effectiveness, adverse events, and toxicity, and side effects associated with synthetic allopathic medications, the existing treatment choices for upper and lower respiratory tract allergic disorders and bronchial asthma have significant limits (Salib et al., 2003). Herbal therapy, like alternative medicine, has regained popularity, efficacy, and safety in the treatment of asthma, as evidenced by controlled clinical trials (Huntley et al., 2000). Ayurveda, an Indian medical system, has documented various medications derived from indigenous plant sources that can be used to treat bronchial asthma and allergy problems. There is a significant prevalence of herbal medicine used to treat this frequent chronic widespread condition (Gopumadhavan et al., 2005; NIH, 1995). The scientific literature supporting the efficacy of herbal remedies, on the other hand, is lacking. There are just a few well-controlled researches that back up herbal treatments' efficacy in the treatment and clinical improvement of asthma patients. The veracity of their popular function in asthma therapy has yet to be proven scientifically (Szelenyi et al., 2002).

Herbal and ayurvedic pharmaceuticals from the plant kingdom have become a topic of global relevance in the last decade, with both therapeutic and commercial

ramifications. The growing usage of herbs has raised severe questions about their quality, safety, and efficacy across the world. As a result, appropriate scientific proof or assessment has become a criterion for herbal health claims approval. As a result, a lot of researchers are attempting to produce anti-asthmatic and anti-IgE medications scientifically using herbal sources. Plants and natural sources account for about 70% of prescription and OTC (over the counter) drugs used to treat a wide range of ailments. Ephedrine, derived from the Chinese herb Ma Huang, was the first modern prescription natural drug for treating asthma. Cromolyn Sodium is a popular plant-based asthma therapy from Khellin (*Ammi visnaga*) that is often found in Egypt (Taur et al., 2011). Asthma and allergies are lifestyle disorders that can be successfully treated with herbs.

1.6 Arthritis and its complications:

Arthritis is an inflammatory, chronic, autoimmune disease that causes swelling, pain and stiffness in joints. Pain, swelling, and stiffness are all symptoms of an autoimmune condition. Its occurrence varies with age. It is a synovial joint inflammation caused by an immune-mediated reaction. It affects roughly 1% of the global population and is a chronic, inflammatory, and systemic autoimmune disease (Pandey., 2010; Potempa et al., 2017)**.** To relieve the symptoms of this condition, allopathic drugs have been recommended, resulting in adverse effects such as gastrointestinal bleeding and bone loss (osteoporosis).

Types of arthritis:

1) **Osteoarthritis (OA):**

The most prevalent chronic joint disease is OA. Degenerative arthritis, degenerative joint disease, and wear-and-tear arthritis are other names used for OA. A joint is the section where two bones connect. A cartilage acting as a protection sheath that borders the ending of bones wears away with OA, causing joint bones to be rubbed together. This may result in stiffness, pain, and other unpleasant symptoms. OA can occur in joints of the hands, fingers, shoulder, knees and hips. OA is more likely to occur in elderly persons; yet it can be affecting adults of varied age groups (Glyn-Jones et al., 2015).

2) Psoriatic Arthritis:

Psoriasis (skin inflammation) and arthritis (joint inflammation) are symptoms of this disease (arthritis). Reddish, white, patchy, and raised regions of inflammatory skin with scales are symptoms of psoriasis. The scalp, the points of elbow and knees, the navel, and the skin surrounding genital regions or anus are commonly affected. Barely between 10-30% of those who have psoriasis will develop psoriatic arthritis. The onset of the particular form of arthritis is most common between the ages of 30 to 50, yet can also occur earlier in childhood. It affects both men and women equally. Psoriasis, a skin condition, generally manifests first (Ritchlin et al., 2017).

3) Gout:

A kind of arthritis suffering from extreme joint pain, tenderness and redness. Seen when excess of uric acid crystallises and accumulates in the joints, causing pain and inflammation. Gout causes swelling in joints, extreme pain, and redness, mostly in the big toe. Attacks typically during nights can happen all of a sudden. NSAIDs (nonsteroidal anti-inflammatory drugs) can reduce discomfort and minimise the time of an acute

episode. Patients with chronic gout can see reduction in the frequency of episodes by changed habits, such as exercise and diet, drinking less alcohol etc. Patients with persistent gout are frequently prescribed uric acid-lowering medicines (Roddy et al., 2014).

4) Systemic lupus erythematosus (SLE):

The frequently known lupus is SLE. It is an autoimmune illness in which the immune system is seen targeting its own body tissues, that results in wide spread of inflammation and tissue destruction in all the involved organs. Skin, Joints, kidneys, brain, lungs, and blood vessels can be affected. Lupus is not curable, yet it can be controlled with medical interventions and changes in the lifestyle (Dörner et al., 2019).

1.7 Pathophysiology of arthritis:

RA pathophysiology is influenced by varied immune modulator (cytokines and effector cells) and signalling pathways like NF-κB signalling pathway, AMPK signalling pathway jak-stat signalling pathway (Smolen et al., 2003). The joint injury that begins is caused by the intricate interplay of immune modulators. Most IA structures are covered at the synovial membrane (Figure 1.3) (Smolen et al., 2003).
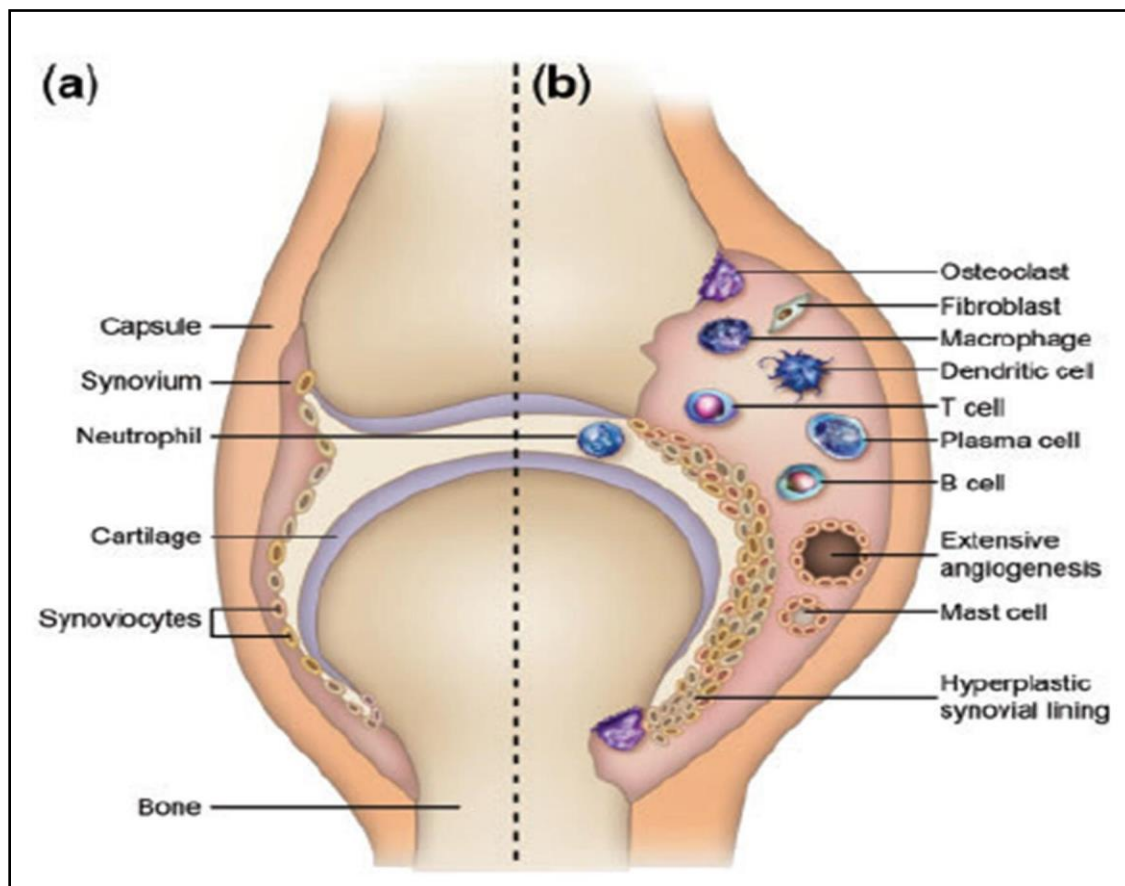
Figure 1.3: Schematic representation of normal joint (a) and joint affected by RA

(b) (Choy et al., 2012)

Synovitis is caused by an influx of localised inflammation. Mononuclear cells (including

T lymphocytes) are activated or both B cells, plasma cells, dendritic cells, and

macrophages are all types of cells. and mast cells) as well as via angiogenesis (Smolen et

al., 2003). The synovium is the connective tissue between the joints. The synovial

membrane swells and produces villi when the lining becomes hyperplastic (Smolen et al.,

2003). Most patients have extra-articular or systemic signs, or both, in addition to joint

symptoms (Hochberg et al., 2008). Rheumatoid nodules, pericarditis, vasculitis, uveitis,

keratoconjunctivitis sicca and rheumatoid lung are examples of extra-articular symptoms (Hochberg et al., 2008). Acute-phase protein production, cardiovascular disease (CVD), osteoporosis, anaemia, depression, and tiredness are examples of systemic symptoms. Activating innate immune response, which involves activating dendritic cells by foreign material and autologous antigens (Figure.1. 4), is the first event in the pathogenesis of RA (Smolen et al., 2003).
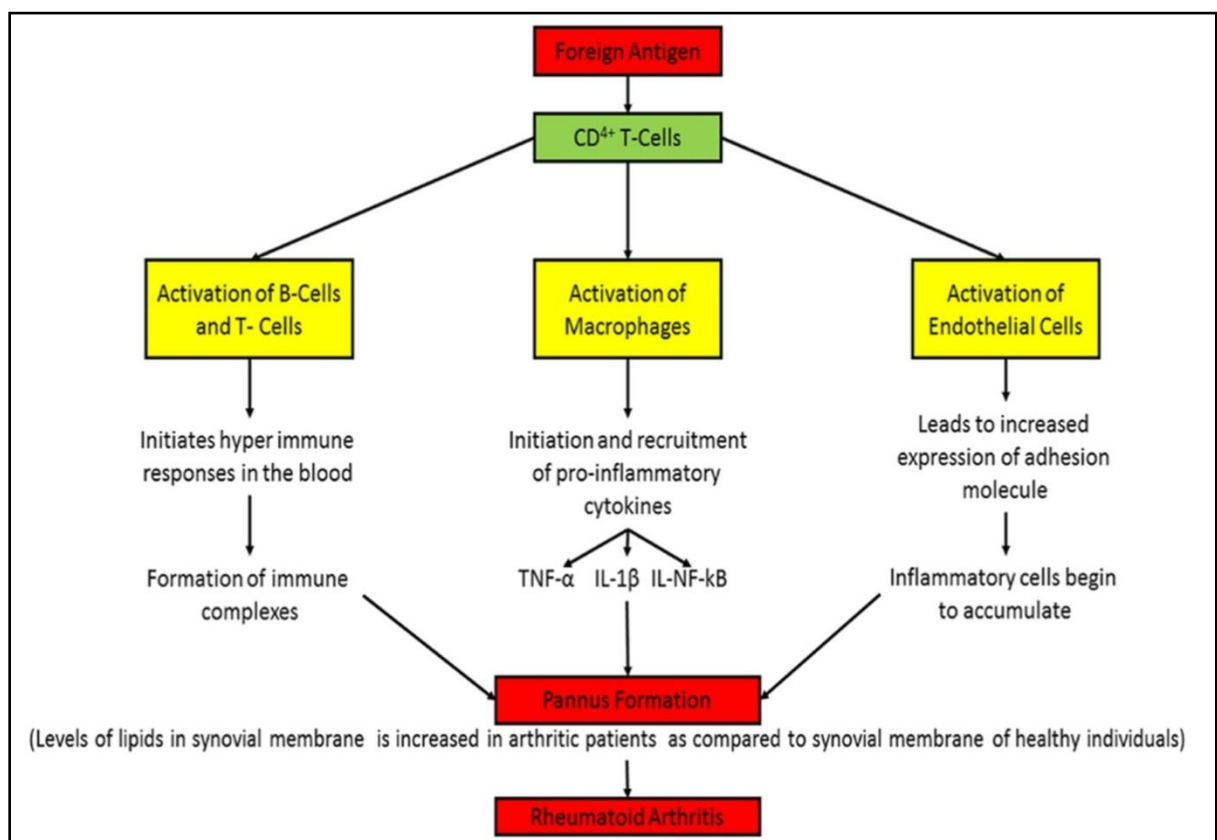
Figure 1.4: Schematic representation of pathogenesis of RA

B cells plays vital part in pathogenesis of RA not only by presentation of the antigens, by production of autoantibodies, antibodies, and cytokines. Autoantibodies to RF and anti-CCP show prevalence in RA patients. Immunoglobulin and differentiation antigens like

CD20 and CD22 are expressed on the cell surface of B cells (Smolen et al., 2007). By complement and Fc-receptor activating, autoantibodies can create bigger immune complexes which promotes the production of pro-inflammatory cytokines, that also includes TNF-a (Smolen et al., 2003). Elevated cytokines and chemokines resulted via activating T- and B-cell, creates a feedback loop leading to increase T-cell, B-cell, and macrophage interacting with each other (Smolen et al., 2003, 2007). Macrophages shows implication in osteoclastogenesis and is a preliminary cytokine such as TNF-a, in addition to antigen presentation (Smolen et al., 2003). Pro-inflammatory cytokines (e.g., IL-6 and TNF-a) are known to have a role in pathophysiology of RA (Smolen et al., 2003, 2007). TNF-a and IL-6 plays the most important role in RA pathobiology, also IL-1, VEGF, and IL-17 also play their respective parts. These cytokines show activation of genes implicated in inflammatory responses, that includes other cytokines and MMPs involving in tissue destruction (Smolen et al., 2003) this is explored in more detail in the next sections. Th 17 (TH17), an IL-17-secreting fraction of CD4+ cells that plays a key part in synovitis, has currently linked to the development of a number of inflammatory and autoimmune disorders, including RA. The involvement of TH17 cells in SF and peripheral blood of individuals having RA implies that this strong proinflammatory cytokine is involved in the disease's pathogenesis (Zhang et al., 2015; Azizi et al., 2013).

Etiological factors of RA (Alam et al., 2017):

a. **Sex:** Chances of development of RA is more in women in comparison to men.

b. **Age:** RA may develop at any stage but chances are more in middle age individuals.

c. **Family history:** If someone in family has rheumatoid arthritis, individuals in that family are at a higher risk.

d. **Smoking:** Smoking increases the chances of development of RA.

e. **Obesity:** Obese individuals are at higher risk of RA.

1.8 Current treatment of arthritis and drawbacks

Glucocorticoids (GCs), nonsteroidal anti-inflammatory medicines (NSAIDs), biological agents including IL-1 receptor antagonists (IL-1Ra) and TNF-a blockers, and disease-modifying anti-rheumatic drugs (DMARDs) are all utilised to treat pain and inflammation in the joints (Moutsopoulos et al., 2021). However, there is a need to enhance the efficacy and safety of RA medication so that it is more effective and has fewer adverse effects. Several cyclooxygenase-2 inhibitors are removed from the market owing to cardiotoxicity, and DMARDs cause long-term adverse effects such as persistent fungal infections, TB, liver damage, lymphomas, and myelosuppression. Immune mediators IL-6, IL-1ß, and TNF-a are inhibited by biological drugs. Patients are frequently unable to maintain biological therapy or cease their medicine after a little period of usage, despite their availability and expensive cost.

1.9 Role of herbal medicines in the treatment of arthritis:

Because of the toxicity and side effects of allopathic drugs, herbal therapy is becoming more popular. The creation of effective therapeutic medicines relies heavily on medicinal plants. By influencing the expression of pro-inflammatory signals, a variety of medicinal plants are now playing a significant role in the creation of strong therapeutic anti-arthritic medicines. In the case of arthritis, they are utilised for prevention, promotion, and

treatment (Verma et al., 2008). These are some of the more notable cases. In both the nascent and development phases of FCA (Freund's complete adjuvant) produced arthritis, *Strychnos potatorum* extract markedly normalises haematological and biochemical abnormalities in adjuvant caused arthritic rats (Ekambaram et al., 2010). *Sida rhombifolia* shown to be quite beneficial in the treatment of arthritis (Gupta et al., 2009). *Premna serratifolia* has potent anti-arthritic properties in adjuvant-induced arthritis (Rajendran et al., 2010).

Attempts have been undertaken over the last three decades to generate useful anti-arthritic therapy from natural sources, notably plant phytoconstituents. Plants including *Daucus carota, Ammania baccifera, Cyperus rotundus*, *Cleome gynandra,* their pharmacological activity was triggered by inhibiting the numerous types of inflammatory mediators involving in the inflammatory process. We proposed this initiative to investigate potential anti-arthritic agents derived from herbal sources as a result of these hopeful findings. Our goal is to quickly heal such a debilitating condition without the side effects that are now linked with allopathic anti-arthritic medications. Their pharmacological activity was triggered by inhibiting the numerous types of inflammatory mediators involved in the inflammation process. Various research groups initiated to investigate potential anti-arthritic agents derived from herbal sources as a result of these hopeful findings. Their goal is to quickly heal such a debilitating condition without the side effects that are now linked with allopathic anti-arthritic medications. *Hybanthus enneaspermus, Piper nigrum, Premna serratifolia, Sida rhombifolia, Strychnos potatorum*, and *Syzygium cumini,* have been proven to be potential anti-arthritic agents

(Arya et al., 2011). Their pharmacological activity was triggered by inhibiting the numerous types of inflammatory mediators involved in the inflammation process. We proposed this initiative to investigate potential anti-arthritic agents derived from herbal sources as a result of these hopeful findings. Our goal is to quickly heal such a debilitating condition without the side effects that are now linked with allopathic anti-arthritic medications.

Treatments for arthritis include a number of flaws that limit their effectiveness. Herbal medication offers a wide structural variety and excellent potential against RA treatment based on inflammatory biomarkers, which is not typically found with synthetic medicines. Polyphenols, resveratrol, thymoquinone, curcumin, celastrol, gambogic acid, and hesperidin when administered in a dose-dependent way, have a great effectiveness against arthritis. The advantages are ascribed to their ability to target cytokines, chemokines, adhesion molecules, NF-k, and nitric oxide, among other things.

Medicinal plants with antiarthritic potential are summarised in below table 1.

Table 1.1: Summary of antiarthritic plants (Choudhary et al., 2015)

Sr. No	Botanical Name	Family	Traditional Name	Part used
1	*Acacia catechu* Willd.	Fabaceae	Mimosa catechu	Root
2	*Achillea millefolium* Linn.	Compositae	Rojmari, bloodwort, arrow-root	Herb
3	*Adhatoda vasika* Nees.	Acanthaceae	Adarushah	Leaves
4	*Barosma crenulata* Hook.	Rutaceae	Bucchu, buku	Leaves
5	*Bula alba* Linn.	Cupuliferae	White birch bark	Leaves
6	*Cadaba indica* Lamk	Capparidaceae	Indian cadaba	Leaves
7	*Cassia tora* Linn.	Fabaceae	Charota, taga	Leaves
8	*Citrus aurantium* Linn.	Rutaceae	sweet orange	Fruit
9	*Dolichos falcatus* Klein.	Papilionaceae	Kattamara	Seed
10	*Euphorbia nivulia* Ham.	Euphorbiaceae	Katathohar	Leaves

1.9 An Overview of dementia:

Dementia is a term used to describe an acquired worldwide impairment of intelligence, memory, and cognitive skills, as well as difficulty with problem-solving abilities in daily life. Alzheimer's disease (AD) is a progressive neurodegenerative condition

characterising a significant cholinergic deficit in the brain that primarily affects the elderly and is the most prevalent cause of dementia. AD is characterised by intracellular neurofibrillary tangles and extracellular amyloid beta protein (A) depositions in the hippocampal area of the brain, culminating in senile plaques (Chopra et al., 2011). This causes cholinergic neurons to die in the hippocampus, affecting memory and cognitive processes. The breakdown of amyloid precursor protein (APP) by the secretase enzyme (BACE), which is a rate-limiting step, initiates A formation and is a key therapeutic target for decreasing cerebral A levels in the treatment and prevention of AD. Apart from Alzheimer's-related dementias, the progressive accumulation of cognitive impairments in combination with post-stroke, symptoms varying depending on the location of ischemia & stroke is also highly common in today's age, and is connected to diabetes, hypertension, and other factors. Through separation, characterization, and *in vitro*, *in vivo* study for proper efficacy and targeting with high therapeutic window in low dose, a valuable contribution to the experimental medicine area of neurological and cognitive therapeutics could be made for treating dementia safely or as a prophylactic therapy over major first line allopathic drugs such as piracetam, rivastigmine.

Multi-infarct dementia, which is characterised by symptoms of dizziness, memory and learning difficulties, is linked to transient ischemia episodes, cerebrovascular accidents such as stroke, organic psychosyndromes, and other conditions. According to the Delphi consensus research, roughly 81 million individuals throughout the world will be affected by 2040. According to the report, the pace of rise in dementia patients in India, China, and other south Asian and western Pacific countries will be more than 300 percent. In

accordance with the WHO and the Global Burden of Disease Survey, chronic mental stress-related diseases will be the second biggest cause of disability by 2020 (Bohra et al., 2015). According to a 2011, Nielsen poll of 21 nations, Indian women are the world's most psychologically worried individuals, with an 87% rating. Chronic psychological stress and depression are becoming more prevalent in the general population, and they have been related to an increased risk of dementia and cognitive deficits. According to WHO, till 2025, over 75% of the anticipated 1.2 billion individuals aged 60 and up would live in emerging nations such as India and China. Every 20 years, the amount of people with dementia is expected nearly about quadruple, in 2020 about 3 million and in 2040 about 81.1 million. India would have the fastest rate of growth (about 336 percent), which might be a very concerning situation. An increase in the number of persons suffering from age-related dementia has paralleled an increase in life expectancy. In stressful settings, poor learning abilities, reduced memory, poorer retention, and sluggish recall are all prevalent issues. Stress and emotions, as people become older, can cause problems including decreased learning, memory loss, forgetfulness, dementia, and more serious risks like schizophrenia and AD.

1.10 Pathophysiology of Alzheimer's disease (AD):

The hippocampus, amygdala, entorhinal cortex, and cortical association regions of the frontal, temporal, and parietal cortices, also the subcortical nuclei such as the serotonergic dorsal raphe, noradrenergic locus coeruleus, and cholinergic basal nucleus, all tends to show neuronal depletion. Forming of tangles occurs in sequential process, initiating with trans-entorhinal cortex, later entorhinal cortex, the CA1 area of

hippocampus, also ends with the cortical association regions, which is remarkably affected in the frontal, parietal, and temporal lobes. Hence, the number of amyloid plaques, the width and position of tangle developing is seen in association with severe dementia. Depletion of neurons and atrophy in temporofrontal cortex in AD, that results in inflammation and accumulates amyloid plaques, an abnormal cluster of protein fragments, and tangled bundles of fibres. Leading to elevation monocytes and macrophages in the cerebral cortex, along with microglial cell activation in the parenchyma. Pathophysiology of AD is summarized in Figure **5**. In AD and similar tauopathies, tau, a microtubule-associated protein, tends to produce insoluble filaments that collect as neurofibrillary tangles. Tau controls the construction and maintains the structural stability of microtubule under physiological circumstances. However, in the diseased brain, tau becomes abnormally hyperphosphorylated, causing the microtubules to break free and the free tau molecules forming paired helical filaments. The ever-increasing data suggests that tau hyperphosphorylation is due to disrupted cellular signalling, typically because of disrupted balance in various protein kinases and phosphatases activity. The ß-amyloid peptide (Aß) is seen to have a major part in creating this imbalance in AD (Medeiros et al., 2011). The amyloid-beta (Aβ) peptide is a product of the successive proteolytic processing of amyloid precursor protein (APP) by β-secretase and γ-secretase. The origin of AD has been linked to an excessive build-up of a major component of amyloid plaques. Neurogenesis abnormalities in brain have lately been linked to the development of AD. Furthermore, new research suggests that APP may impact the proliferation of neural progenitor cells (NPC) and control the

transcriptional activity of a variety of genes. APP's effect on neurogenesis is provided differentially by its two distinct domains, soluble secreted APPs (sAPPs, mostly sAPP) and APP intracellular domain, according to studies (AICD). AICD was discovered to adversely affect neurogenesis, whereas sAPP was revealed to be neuroprotective and crucial to neurogenesis (Zhou et al., 2011).
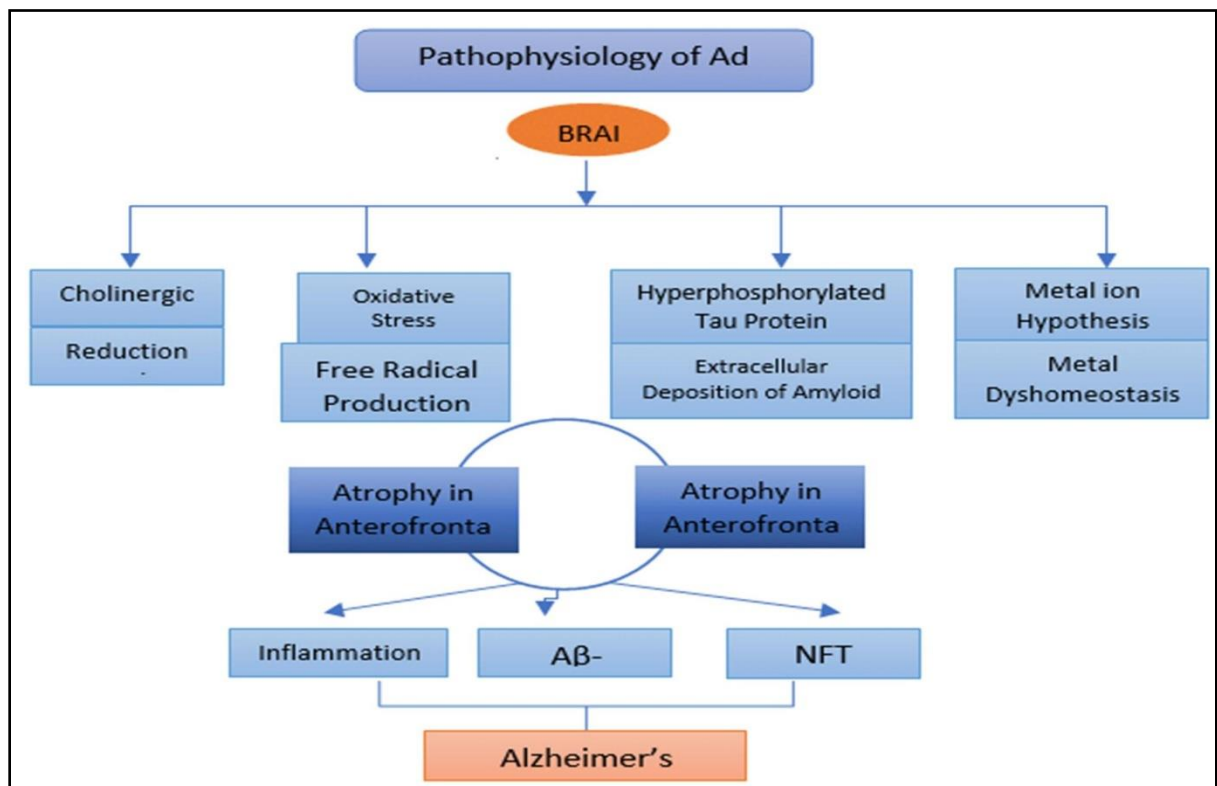
Figure 1.5: Pathophysiology of Alzheimer's disease leading to dementia

1.10.1 Cholinergic reduction:

With minor to severe AD, binding of cholinergic receptor is decreased in different areas of brain, seen to be linked with neuropsychiatric symptoms. Reduced receptor binding might be seen linking to lower speed in elder persons. *In vivo* cholinergic receptor binding may have links to different brain modifications further linking to aging and AD,

also possible target for molecular therapy. Drugs that regulate acetylcholine transmitter level, that includes cholinesterase inhibitors (ChEIs) and donepezil, have been an essential symptomatic therapy for AD for over 20 years (Chase et al., 2017).

1.10.2 Oxidative stress:

In human beings, reactive oxygen and nitrogen species (ROS and RNS) formation is seen normally and pathologically in such processes. They tend to show double action in which they both have beneficial action in cellular signalling pathways and poisoning actions harming the cellular build-ups (cell membrane, lipid, protein, and DNA). The higher consumption of O_2 by brain of about 20 % of mitochondrial respiratory tissues, makes it prone to oxidative stress to a greater extent. The neuron, including a high amount of polyunsaturated fatty acids, functioning unit of brain. It can show interaction with ROS, that causes lipid peroxidation and molecular apoptosis; oxidative stress damage is a result of absence of glutathione in neurons (Liu et al., 2017).

1.10.3 Hyperphosphorylated tau protein and amyloid β protein:

The development of senile plaques (SP), that is formed by amyloid beta (A) deposition, is an essential pathological characteristic of AD. Generally, Aβ are soluble short peptides is produced by the action of β-secretase and γ-secretase on the precursor protein of amyloid (APP). It depends on oligomerization, that the disrupted balance between β amyloid (A) generating and clearing results in various forms of harmful oligomeric, such as protofibrils, fibrils, and plaques. Even though the cause for Aβ's production is not yet known, the sequence, concentration, and circumstances of Aβ's stable features are all vital features (Liu et al., 2017). Various types of variables linking to the pathogenesis of

AD, that includes amyloid/tau toxicity, cholinergic dysfunction, and oxidative stress/mitochondrial dysfunction (Mohamed et al., 2016).

1.10.4 Metal ion hypothesis:

Metal dyshomeostasis has a part in the development stages and pathogenesis of a various diseases, like neurodegenerative disorders and cancer. The ionosphere and metal chelators are identified modulators of transition metal homeostasis, also few of them have importance in therapeutics. Drugs targeting transition metal homeostasis are not restricted to metal-binding molecules. It is evident that balance for redox transition metals, namely iron (Fe), copper (Cu), and other trace metals, has been changed. In AD, their quantities in the brain are elevated. Copper, manganese, aluminium, and zinc are all seen to be involving in varied neurological diseases (Weekley et al., 2017).

1.11 Current treatment of Dementia and drawbacks:

Patients suffering from different dementias such as transient ischemic attacks, stroke, organic brain syndromes, mental retardation, multi-infarct dementia, and others, ranging in age from childhood to the elderly, now rely on restricted medication therapy choices. There is yet to be a notable therapeutic success that prevents, alters, or manages dementia; instead, present dementia therapies are primarily symptomatic. Cholinesterase inhibitors are the only FDA-approved first-line pharmacologic therapy for Alzheimer's disease, and they have been demonstrated to be successful in treating the cognitive, behavioural, and intellectual impairments associated with the disease. Donepezil, rivastigmine, and galantamine are the most common AChE inhibitors used to treat cholinergic insufficiency in the brain, although they have limitations such as short

duration of action, limited bioavailability, and a restricted therapeutic index. Furthermore, certain synthetic drugs touted as cognition enhancers, such as piracetam, amphetamine, pemoline, pyritinol, and others, are not suitable for long-term use in humans due to extra adverse effects and non-specificity in site of action. Furthermore, major side effects such as hepatotoxicity restrict its use.

1.12 Plant based phytochemicals for the Dementia:

Preclinical models of dementia, depression, and cerebral ischemia-related cognitive impairment show that plant-based phytochemical research from a variety of historically used medicinal herbs is extremely helpful. Galantamine, a plant-based isolated chemical from *Galanthus woronowii*, is a current FDA-approved AChE inhibitor for AD-related dementia. Other plant-based AChE inhibitors, such as Physostigmine from the calabar bean *Physostigma venenosum* and Huperzine A from *Huperzia serrata*, have been identified in animal and human trials to be possible lead candidates for treating dementia by enhancing central cholinergic activity. The neuroprotective potentiality of numerous flavonoids, polyphenolic, and glycosidic chemicals found in plant reservoirs, which have been studied in animal models, indicates a better treatment outcome in dementia-related disorders in the future. Bacosides from *Bacopa monnieri*, often known as Brahmi, an Indian traditional medicinal herb, have been shown in several experimental and clinical investigations to improve learning and memory retention. Consumption of a diet high in plant polyphenols has been shown to reduce cognitive decline and stress in people and animals (Joseph et al., 2005; Spencer et al., 2010). Flavanols, a subclass of plant polyphenols with proven pleiotropic roles in neuroprotection, cognition (Spencer et al.,

2010), and mood (Sokolov et al., 2013), are found in a variety of interventions such as cocoa, green tea, blueberries, grapes, and others (Vignes et al., 2006).

As a result, traditional medicines are being rediscovered in order to implement a therapeutic approach. In addition, antioxidants, anti-inflammatory, anti-apoptotic, neurotrophic, and anti-amyloid/genesis medicines have all been investigated in AD *in vitro* and *in vivo* models. Natural dietary polyphenols including curcumin, resveratrol, and catechins have gotten a lot of interest as promising possibilities for Alzheimer's treatment. These polyphenolic chemicals have been shown to protect against AD by acting as antioxidants, anti-inflammatory agents, and anti-amyloidogenic agents, as well as stimulating cellular stress adaptive responses known as the "neurohormesis" process. *Ginkgo biloba* extract Egb761 has been the most extensively examined, as previously mentioned. Huperzine A, a cholinesterase inhibitor derived from *Huperzia serrata*, has also been shown to be beneficial in treating AD. Herbal treatments are thought to be helpful and safe for treating AD, according to a systematic analysis of clinical data. In this light, both scientists and the pharmaceutical sector have challenges in developing novel molecules that are potent enough to act on a variety of biochemical targets while still being low in toxicity. The quest to develop cures for this terrible disease, on the other hand, has reignited interest in natural chemistry. Plant active ingredients, well-known herbs, and their extracts may lead to the development of viable treatments for AD. It is widely assumed that natural product chemistry research contains a huge number of untapped potentials that might open up new avenues for treating AD.

Medicinal plants with anti-Alzheimer's potential are summarised in below table 2.

Table 1. 2: Summary of antidementia plants (Perry et al., 2011)

Sr. No	Botanical Name	Family	Traditional Name	Chemical constituent
1	*Ginkgo biloba* L.	*Ginkgoaceae*	Kew tree	Quercetin, kaempferol, isorhamnetin
2	*Huperzia serrata*	*Lycopodiaceae*	Toothed clubmoss	Alkaloids: huperzines A and B
3	*Panax ginseng*	*Araliaceae*	Ashwagandha	Triterpenoid saponins: ginsenosides
4	*Polygala tenuifolia Willd.*	*Polygalaceae*	Yuan Zhi.	cinnamic acid
5	*Crocus sativus* L.	Iridaceae	Saffron	Carotenoids: crocin
6	*Vinca minor* L.	Apocynaceae	Lesser periwinkle	Alkaloids: vincamine
7	*Melissa officinalis* L.	(Lamiaceae	Lemon balm/melissa	Essential oil; rosmarinic acid and derivatives
8	*Leucojum aestivum* L.	Amaryllidaceae	Daffodil/narcissus	galantamine

2. Literature review

2.1 Literature review of medicinal plants used in asthma:

A current state for allopathic asthma therapy is filled with complications. Isoprenaline, salbutamol, theophylline, and other anticholinergics, as well as corticosteroids, cause a variety of side effect, including muscle tremor (dose-related), tachycardia, dry mouth, throat irritation, respiratory depression, restlessness, nervousness, sedation, weight gain, dizziness, nausea, and ankle edema (Tripathi et al., 2001). Ephedrine, derived from the Chinese herb Ma Huang, was the initial modernized drug used for treating asthma. Cromolyn Sodium, a popular asthma therapy made using Khellin (*Ammi visnaga*) plant located in Egypt (Taur et al., 2011). Asthma and allergies are lifestyle disorders that can be successfully treated & managed with herbs & spices as well as ethnic medicinal plants.

One study reported synergistic herbal composition to treat bronchial asthma comprises herbs like *Achyranthes aspera, Woodfordia furiticosa, Solanum xanthocarpum, Justicia adhatoda, longa, Holarrhena antidysenterica, Wnicostemma littorale, Calotropis procera, Piper nigrum, and Elettaaria cardamomum.* Individuals with reversible asthma, a randomised double-blind placebo-controlled clinical trial for 12-week was undertaken for comparing effectiveness of a plant-derived preparation (DCBT4567-Astha-15) to orally taken salbutamol, salbutamol with theophylline, also a matched placebo. The treatment of reversible asthmatics, DCBT4567-Astha-15 seen effective just like salbutamol (12 mg/day) or salbutamol (12 mg/day) in conjunction with theophylline (200 mg/day). Patient's quality of life increased as a result of the DCBT4567-Astha-15 medication therapy (Murali et al., 2006).

According to Kumar et al., *Aervalanta* Linn. has antiasthmatic properties via suppressing mast cell degranulation. They looked at the effects of ethanol extract of *Aerva lanata* aerial parts at 100 g/ml *in-vitro* in the isolated goat trachia chain formulation paradigm and 30 & 60 mg/kg dosages oral *in-vivo* in mice with mast cell degranulation and clonidine-induced catalepsy. The extract had anti-asthmatic action that was dose dependant (Kumar et al., 2009).

Using specialised *in-vivo* animal models, extracts of the *Actiniopteris radiata* plant were tested for their therapeutical potency as an anti-stress and anti-allergic agents in asthma. Only the ethanolic extracts (AREE) at increased dosing with 100 mg/kg IP (intraperitoneally) substantially ($p<0.05$) reduced milk-induced eosinophilia in comparison with the control group, whereas even lower doses of 50 mg/kg IP inhibited milk-induced leucocytosis in mice. Other extracts, like methanol, ethyl acetate, and petroleum ether were unable to achieve the same level of potency. Because of the presence of several polar secondary metabolites in the ethanol extract, the results obtained confirm the traditional claim of *Actiniopteris radiata* is useful for asthma. (Vadnere et al., 2013).

According to Youssouf et al., 2007, *Euphorbia hirta*, a common asthma herb, demonstrated promising antiasthmatic effects by inhibiting passive cutaneous and paw anaphylactic reactions and protecting mast cell degranulation (Youssouf et al., 2007).

In-one of the studies isolated guinea pig ileum, isolated guinea pig tracheal chain, & histamine-induced bronchoconstriction in guinea pig, the anti-histaminic action of ethanol extracts and hydro distilled extracts of leaves of *Piper betel* Linn. was tested pharmacologically. Both extracts (100µg/ml) effectively decreased histamine-induced

dose-dependent contractions of guinea pig tracheal chain and isolated guinea pig ileum prepared in the current investigation ($p < 0.05$). In both cases, the ethanolic extract was less efficacious than the hydro distilled extract of *Piper betel*. Ethanol extracts (100µg/ml and 200µg/ml) and hydro distilled extracts (100µg/ml and 200µg/ml) were reported for protecting guinea pigs alongside histamine-induced bronchoconstriction ($p < 0.001$). When comparing to the ethanolic extract of *Piper betel* the hydro distilled extract (200µg /ml) was more effective (Jawale et al., 2009).

Tamarindus indica (Caesalpiniaceae) is used traditionally in treating asthma, diarrhoea, uterine and vaginal symptoms, inflammatory issues, and a range of various ailments. This investigation includes a methanol extract of *Tamarindus indica* leaves at dosages of 175, 350, and 700 mg/kg, p.o., considerably decreased ($p < 0.01$) milk-induced leucocytes and eosinophilia in rats and significantly inhibited ($p < 0.01$) milk-induced leucocytes & eosinophilia in mice. Clonidine, an agonist of the a2 adrenoreceptors, causes catalepsy in mice that are blocked by histamine H1 receptor antagonists. The clonidine-induced catalepsy in mice was substantially decreased ($p < 0.01$) after treatment with a methanol extract of *Tamarindus indica* 250, 500, &1000 mg/kg, p.o. doses. In laboratory mice, the methanolic extract of *Tamarindus indica* leaves displayed considerable antihistaminic, adaptogenic, and mast cell stabilising activities, according to the findings (Tayade et al., 2009).

Strychnos potatorum has anti-anaphylactic properties. Linn seed extract was tested for anaphylaxis using compound 48/80, and mast cell stabilisation was investigated using rat peritoneal mast cells. The chloroform, petroleum ether, and methanol extracts were shown to be strong and had substantial inhibitory effects on compound 48/80induced anaphylactic response and mast cells activating in this investigation. This chemical

also greatly reduced the amount of nitric oxide produced by compound 48/80 in rat peritoneal mast cells. *S. potatorum* seed extracts show substantial antianaphylaxis action by stabilising mast cells and inhibiting nitric oxide production (Savali et al., 2010).

Methanolic extracts anti-asthmatic property of *P. incarnata* leaves was tested in guinea pigs versus acetylcholine chloride (Ach)-induced bronchospasm. The rat's treatment with 100 mg/kg dosage of the extracts showing a substantial decrease in dyspnoea-related convulsions after a seven-day treatment regimen. The 50 mg/kg dose had no preventative impact, and the preventive benefits against Ach-chloride-induced dyspnoea was likewise diminished to a larger dosing of 200 mg/kg. This might be attributed to problems with an alpha-adrenoceptor function that have been described following long-term or excessive usage of alpha-receptor agonist (Dhawan et al., 2003).

In unrestraining guinea pigs treated using histamine, a standardised aqueous extract (AE) and a pure fraction (BuF) of *Cecropia glaziovi* leaves was examined. A full body plethysmograph was used to record changes in breathing pressure and rate before and after therapy. Later by treating by semi-purified procyanidin/flavonoids enriched BuF (0.1 g/kg p.o.), the amount of histamine required to cause bronchospasm was raised five times, and two-fold after treatment with AE (1.0 g/kg p.o.). Previous therapy using propranolol (10.0 mg/kg i.p.) prevented both effects. *In vitro* incubating BuF (0.1-1.0 mg/ml) reduced the maximum responses of guinea pig trachea muscle towards histamine by 13-55 percent without affecting the EC50. The findings corroborated previous reports on *Cecropia* extract beneficial pulmonary

effects. Bronchodilation *in vivo* seen linked to beta-adrenergic action, which was only seen *in vitro* at higher doses of pure extracts (Delarcina et al., 2007).

Curcuma aeruginosa is one of the medicinal herbs useful for treating asthma (Zingiberaceae family). *C. aeruginosa* extracts were examined for spasmolytic action in different organs of guinea pig trachea. In comparison with negative control, the reduction in spasmolytic activities in guinea pig trachea caused by *C. aeruginosa* extract seen much superior (*p = 0.022*). Extracts may show potential needs to explored in future for using as novel & naturally found source of antiasthma medicines (Paramita et al., 2018).

Euphorbia thymifolia (Euphorbiaceae) is useful in treating cough, bronchial asthma, bleeding piles, and diarrhoea according to Ayurveda. Methanolic and aqueous extracts of *E. thymifolia* (ET) were tested for experimenting animals for anti-anaphylactic, mast cell stabilising, and antiasthmatic activities. Treatment with *E. thymifolia* extract resulted in a substantial reduction in asthma score, as well as normalisation of elevated DC, WBC counts, TNF-, serum IgE, IL-4, and IL-5 in BALF. The protective effect of *E. thymifolia* extract was further substantiated by a histological examination. The number of degranulating mesenteric mast cells were reduced significantly after pre-treatment with *E. thymifolia* extract. The use of *E. thymifolia* extracts resulted in a considerable increase in pre-convulsive dyspnoea (PCD) time. As a result of these observations, it was determined that *E. thymifolia* might be utilized to successfully treat anaphylaxis and asthma (Parmar et al., 2019).

Syzygium cumini (Myrtaceae), often known as jamun, is a widely used medicinal plant that is native to tropical America and Australia and is used to cure a variety of disorders such as diabetes, asthma, inflammation, and others. In animal models, the

anti-inflammatory & anti-asthmatic effect of *Syzygium cumini* ethanol extract along with its phytoconstituents was examined. The extract possesses anti-inflammatory properties, as evidenced by its ability to prevent carrageenan-induced paw edema. In histamine-induced bronchoconstriction (*in vivo*) and mast cell degradation methods, also resulted in a considerable reduction in bronchoconstriction (*in-vitro*) (Alekhya et al., 2020).
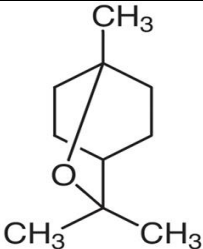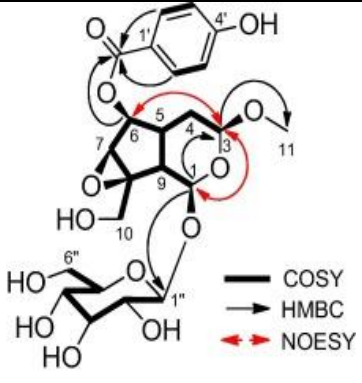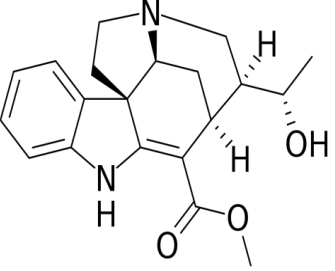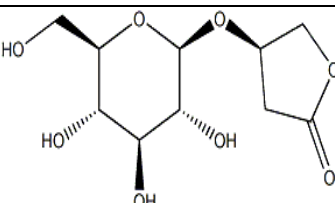
In one of the studies, Soxhlet extraction of the various bark extracts with petroleum ether, butanol, ethyl acetate, and alcohol was performed. Using histamine to induce bronchoconstriction in guinea pigs, these extracts were tested for antiasthmatic and spasmolytic action in isolated guinea pig lung strips. The impact of *Ficus racemosa* bark extracts on an isolated guinea pig lung strip were investigated to figure out how the extracts exerted muscular relaxant action. Few extracts were found to be helpful against histamine-induced contractions in the investigation. The presence of saponins & flavonoids inside the extracts might be accountable for their antiasthmatic effect, according to the findings (Saleem et al., 2019).
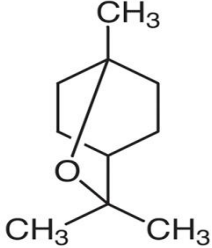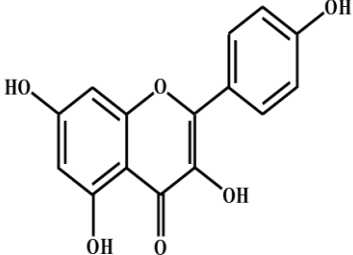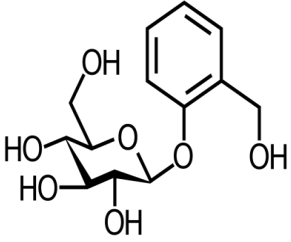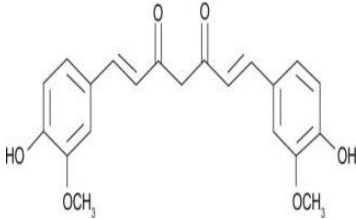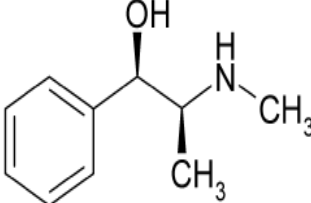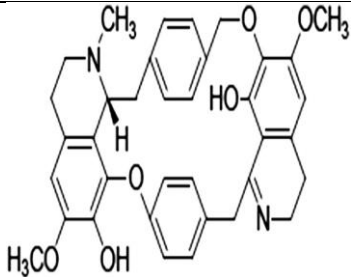
The aerial portions of a *Barleria prionitis* plant were tested for antiasthmatic action and active moieties were separated. The anti-inflammatory investigation was conducted on adult Wistar albino rats. On isolated goat trachea, histamine-induced bronchospasm was studied. The anti-inflammatory action of carrageenan was found to be dose-dependent in a rat paw edema model. The methanol extract of aerial portions of *Berleria prionitis* substantially decreased the histamine-induced dose-dependent constriction of the goat tracheal chain (*p<0.001*). The antihistaminic (H1 receptor antagonist) action of *Berleria prionitis* methanol extract (MEBP) is substantial (Manekar et al., 2018).
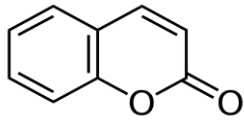
In one of the studies, of Lignosus *rhinocerus* (*L. rhinocerus*), was found to be efficacious for treating ovalbumin-inducing airway inflammation in rodent model of asthma. By usage of gas chromatography- mass spectrometry, volatile ingredient of *L. rhinocerus* hot water extracts were examined in this work (GC-MS). On ovalbumin (OVA)-sensitized asthmatic SD rat, the possible anti-asthmatic properties of *L. rhinocerus* extract were explored further. The levels of helper-T 2 cytokines in bronchoalveolar lavage fluid (BALF) and immunoglobulin E (IgE) in the blood, eosinophil infiltrations inside the lungs, as well as including interleukin (IL)-4, IL-5, and IL-13, was identified. The extract efficiently inhibited eosinophil amount in BALF whereas reducing eosinophil infiltrating in lungs (Johnathan et al., 2016), as well as the raising total IgE in serum & IL-4, IL-5, and IL-13 levels in BALF.

The anti-asthmatic efficacy of aqueous and methanol extracts of the combination herbs *Oregano* (*Coleus amboinicus Lour*) and *Yerba buena* (*Mentha arvensis*) and in asthma-induced mice was examined utilising IgE as a criterion in one of the research projects. The IgE levels in mice treated with aqueous and methanol were reduced by 50% and 60%, respectively ($p = 0.018$). In mice, extract had considerable ($p = 0.001$) anti-inflammatory action, proving its influence on IgE. The potential impact of the extract was further demonstrated by the enlargement of the alveoli in treated mice, according to lung histology. Aqueous and methanolic extracts of Yerba buena and Oregano might show promising health advantage for asthma (Robles et al., 2017).

Table 2.1: Potential phytochemicals and their mechanism of actions (Amaral-Machado et al., 2020)

Sr. No	Plant source	Active compound	Chemical structure	Mechanism of action
1	*Eucalyptus globulus*	1,8-Cineol		Reduction in expressing NF-κB target gene MUC2
2	*P. rotundum* var. *subintegrum*	3-Methoxy-catalposide		Inhibition in expression of proinflammatory genes (IL-6, IL-1β, and TNF-α), nitric oxide synthase (iNOS), & cyclooxygenase (COX)-2
3	Leaves of *Alstonia scholaris*	Scholaricine		Reduction in the expressing eotaxin and serum IgE, inflammatory cytokine (IL-4) & reduce eosinophilia
4	*Anoectochilus formosanus Hayata*	Kinsenoside		Reduce IL-4 by Tregs and enhance IL-12 and IFN

5	*Artemisia maritime*	camphene, 1,8 Cineol, *β-caryophyllene* and, camphor		Inhibition of Ca^{2+} channels and phosphodiesterase activities
6	*Aster tataricus*	Kaempferol, aurantiamide, and astin C		Inhibition of NF-κB & promotes in activating beta-2 adrenergic receptor
7	*Baliospermum montanum Müll. Arg. (*Euphorbiaceae*)*	Alkaloids, glycosides diterpenoids, and triterpenoids,		Stabilization of mast cell degranulation & reducing histamine release
	Boswellia serrata, Glycyrrhiza glabra, and *Curcuma longa*	fl-boswellic acid and curcumin		Reduction in plasma levels of nitric oxide, leukotriene C4, and malondialdehyde
8	*Carica papaya*	Alkaloids, steroids, and quinines and tanins,		Reduction in IL-4, IL-5, TNF-α, NF-κB, eotaxin, and iNOS
9	*Cissampelos sympodialis Eichl*	Warifteine		Reduction of IL-3 and IL-5, increasing the IL-10 levels, and reduce inflammatory

				cells density
10	*Citrus tachibana*	Coumarins, flavonoids, and carotenoids		Control Th1/Th2 imbalance via inhibiting NF-κB signalling

2.2 Literature review of medicinal plants used in arthritis:

By influencing the expression of pro-inflammatory signals, a variety of medicinal plants are now playing a significant role in the creation of strong therapeutic anti-arthritic medicines. In the case of arthritis, they are utilised for prevention, promotion, and treatment (Verma et al., 2008). These are some of the more notable cases. The developmental and developed phase of FCA (Freund's complete adjuvant) both caused arthritis, *Strychnospotatorum* extract markedly normalises biochemical and haematological abnormalities in adjuvant generated arthritic rats (Ekambaram et al., 2010). *Sidarhombifolia* is effective in treating arthritis (Gupta et al., 2009). *Premnaserratifolia* has potent anti-arthritic properties in adjuvant-induced arthritis (Rajendran et al., 2010). Attempts have been undertaken over the last three decades to generate useful anti-arthritic therapy from natural sources, notably plant phytoconstituents. Plants like *Ammaniabaccifera, Daucuscarota, Cleome gynandra, Cyperusrotundus, Hybanthusenneaspermus, Piper nigrum, Premnaserratifolia, Syzygiumcumini, Sidarhombifolia, and Strychnospotatorum* have been discovered to be potential anti-arthritic agents (Arya et al., 2011). Their pharmacological activity was triggered via inhibition of numerous types of inflammation mediator involved in the inflammation process.

Pharmaceutical preparation containing a crude extract of *Plectranthus amboinicus* is beneficial for treating rheumatoid arthritis (Punet et al., 2020). Furthermore, an US patent exists which has reported that poly-herbal compositions are very much used in treating arthritis (Palpu et al., 2008).

Calophyllum inophyllum (Clusiaceae) is a plant that is seen to be in use for long periods to cure pain, inflammatory problems, eye problems, and rheumatism. In Freund's adjuvant-induced arthritic Wistar albino rat model, the antiarthritic action of ethanolic extract of seeds (ESCI) and stem bark (ESBCI) of *Calophyllum inophyllum* was investigated. Clinical, biochemical, histological, and radiological investigations revealed that ESBCI and ESCI had strong antiarthritic action (Perumal et al., 2017).

In rats, the anti-arthritic efficacy of ethanolic extract of *Cardiospermum halicacabum* leaves for doses 125 mg/kg and 250 mg/kg on Freund's complete adjuvant (FCA) induced arthritis was investigated. Various haematological indicators like total erythrocytes, haemoglobin (Hb), erythrocyte sedimentation rate, leucocyte count is used to evaluate the therapy (ESR). The extract decreased FCA-induced arthritis in a dose-dependence manner, with the impact being highly significant *(p<0.001)* at 250 mg/kg. When compared to FCA-induced arthritic animals, extract administration improved body weight considerably. Indomethacin (10 mg/kg) was used to compare the findings. According to the findings, ethanol extracts of *Cardiospermum halicacabum* leaves has a substantial anti-arthritic effect (Kumar et al., 2015).

One of the research goals was to prove scientifically that the ethanol and aqueous extract of *Dissotis thollonii* leaves show anti-inflammatory and antiarthritic capabilities. *In vitro*, anti-inflammatory properties were assessed for protein denaturation, cyclooxygenase, extracellular ROS production, 5-lipoxygenase, and cell

proliferation using inhibition tests; *in vivo*, antiarthritic properties were assessed by the zymosan A-induced monoarthritic test and the CFA-induced polyarthritis model in rats. In vitro research has demonstrated that *D. thollonii* inhibits protein denaturation, inhibits 5-LOX, inhibits COX, and inhibits ROS, while *in vivo* investigations indicated that the plant exhibits antarthritic efficacy in a zymosan-induced monoarthritic model and CFA-induced polyarthritis model in the rats. Findings support use of the plant treating chronic inflammatory illnesses and suggest that it might be important to discover novel anti-inflammatory and anti-arthritic drugs (Djuichou et al., 2019).

The anti-arthritic effect of methanol extract of *Cyathocline purpurea* (MECP) was investigated in rats with Freund's complete adjuvant (FCA)-induced arthritis. In comparison to arthritic control group, MECP demonstrated anti-arthritic activity evident as reduction in joint diameter, paw volume, and rise in paw withdrawal latency, pain threshold, body weight and mechanical nociceptive threshold. MECP (400 & 200 mg/kg) has anti-arthritic efficacy by boosting RBC and Hb levels and lowering WBC, platelets, serum C-reactive protein (CRP), and Rheumatoid factor levels (RF) (*P<0.001* and *P<0.01,* respectively) (Bihani et al., 2014).

The antiarthritic activity of hydroalcohol extracts of *Amaranthus roxburghianus* (HARE) was studied in albino wistar rats. In albino Wistar rats, two dosages of HARE (200 and 400 mg/kg) were evaluated alongside formaldehyde-induced acute non-immunological arthritis and FCA-induced chronic immunological arthritis. In both acute and chronic settings, a dose-dependent and substantial decrease of edema. For the FCA-induced arthritis model, the extract at 400 mg/kg demonstrated the much more powerful and significant (*p<0.05*) inhibition of paw edema, that is corroborated

by findings of paw volume and paw diameter, as well as haematological parameters. HARE preserves the synovial membrane by enhancing health, and it has anti-arthritic potential. As a result, the traditional usage of A. *roxburghianus* for arthritis is supported (Chikatipalli et al., 2020).

In Thailand, *Alternanthera bettzickiana* is utilised a traditional treatment for arthritis. One of the research projects looked at the antiarthritic potency of *A. bettzickiana* ethanol extract (ABEE). Plant characterisation, molecular docking, *in vitro* and *in vivo* research, as well as real-time polymerase chain reaction (RT-PCR) analysis and enzyme-linked immunosorbent assay (ELISA), were carried out to investigate the antiarthritic impact. A significant interaction between these chemicals and cyclooxygenase (COX) enzymes were discovered using molecular docking. The extract reduced paw edema and arthritic scoring decreased cachexia and improved biochemical and haematological changes substantially. The antiarthritic activity of ABEE was further validated by radiographic and histological investigations. The findings revealed that *A. bettzickiana* has antiarthritic properties, confirming its folklore usage for rheumatoid arthritis treatment (Manan et al., 2020).

In the northeaster part of India, *Spondias mangifera (S. mangifera)* fruits are used to treat rheumatism. The ethanol fraction (EtOH-F) of *S. mangifera* fruit extract was investigated for its anti-arthritis and anti-inflammatory properties. At 500 g/mL, in vitro tests revealed a concentration-dependent decrease in protease inhibitors, albumin denaturation, and scavenging activities. In comparison to sick animals, administrating S. *mangifera* alcoholic fraction by above-mentioned dosing showed substantial decrease ($p = 0.01$) in TNF-α, arthritis score, paw diameters, and IL-6. The alcoholic fraction of *S. mangifera* extract have anti-inflammatory and anti-

rheumatoid arthritis properties, and is maybe exploited as a potential agent for innovative target-based therapeutics for managing arthritis (Khalid et al., 2021).
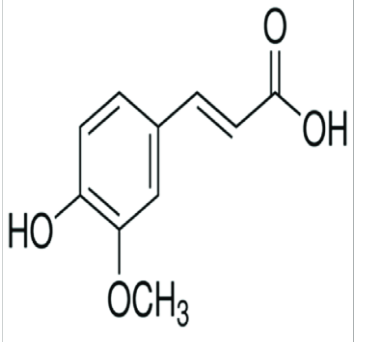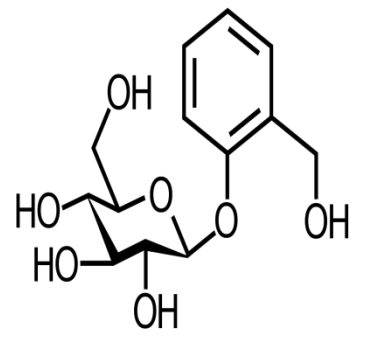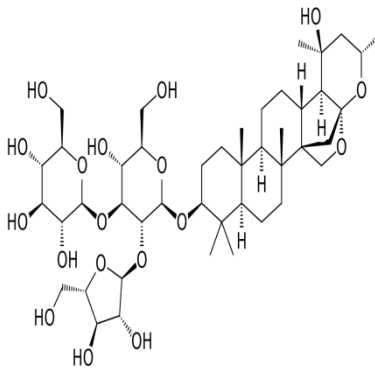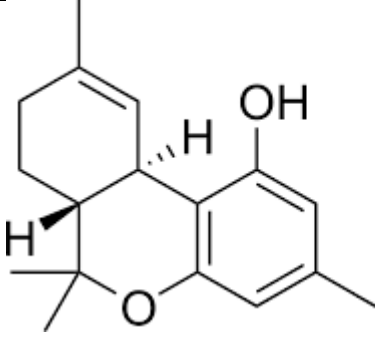
The anti-arthritic potency of *P. braunii* roots was tested *in-vitro* and in-vivo. Using *in-vitro* protein denaturation, membrane stabilisation, and anti-trypsinase tests anti-arthritic activity of extracts was demonstrated. Furthermore, extracts with promising *in vitro* anti-arthritic potential were evaluated orally against formaldehyde-induced arthritis in Wistar rats at 150, 300, and 600 mg/kg/day. The plant's methanolic, aqueous, and ethyl acetate extracts showed notable anti-arthritic properties *in vitro*, as well as a dose-dependent reduction in formaldehyde-induced paw edema. Methanol and aqueous extracts inhibited paw edema and arthritic indices the most ($p<0.05$), lowered elevated levels of platelets and leukocytes, and increased haemoglobin and body weight in arthritic rats. The plant extracts' anti-arthritic action may be due to protein denaturation inhibition and lysosomal membrane stability. The herb showed promising anti-arthritic properties (Saleem et al., 2019).
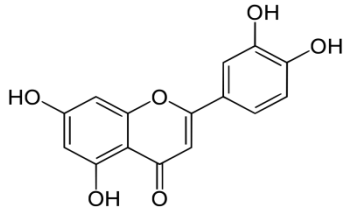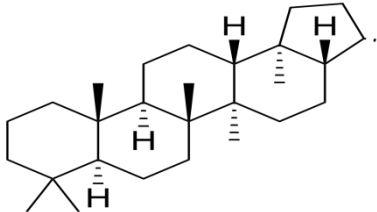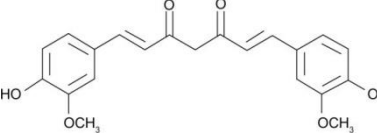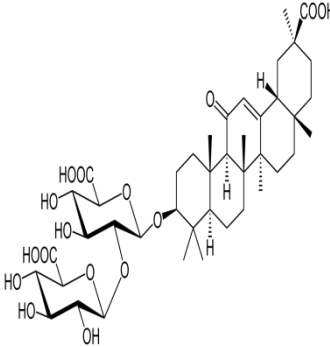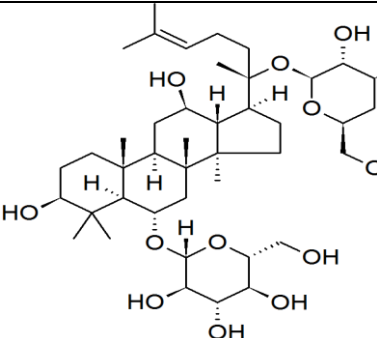
In a rat model of Complete Freund's Adjuvant (CFA)–induced arthritis, the anti-arthritic action of methanol leaf extracts of the plant was studied and made comparison to untreated control and ibuprofen–treated groups. CFA was injected subcutaneously into the right paw to cause arthritis. In comparison to the control, extract decreases arthritis-induced paw volume and joint deformity by a dose-dependence manner. Furthermore, the extract elevates the packed cell capacity significantly [$p<0 .05$]. The study backs up the traditionally used *Urtica pilulifera* for rheumatoid arthritis and inflammatory illnesses and advises that its role in boosting red blood cells be investigated further (Abudoleh et al., 2011).
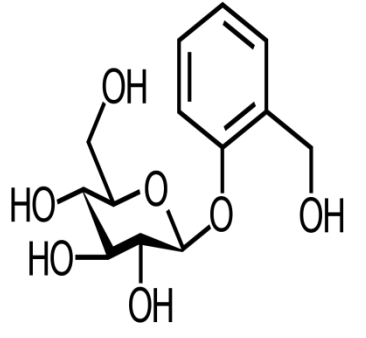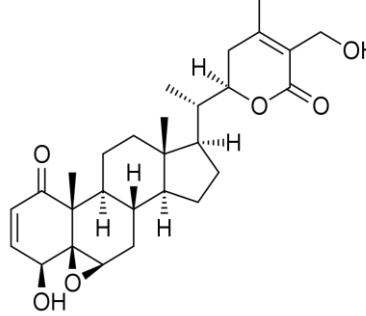
The anti-arthritic activity of the alcoholic extract of the herb Justicia gendarussa was evaluated using Freund's adjuvant-induced and collagen-induced arthritic rat models. The rats were given an ethanolic extract of *Justicia gendarussa* as well as a regular dose of aspirin. As a consequence, the ethanol extract of *Justicia gendarussa* demonstrated anti-arthritic action that was comparable to aspirin statistically. The findings indicate the alcoholic extract of Justicia gendarussa has anti-arthritic properties (Paval et al., 2009).

Incomplete Freund's adjuvant (CFA) induced RA in rats, *Cleistopholis patens* (SBCP) ethanolic and aqueous extracts of stem were investigated for its anti-arthritic potentials. Rheumatoid arthritis induction resulted in a substantial *($p < 0.05$)* rise in paw size, inflammatory markers, and MDA, as well as a significant *($p < 0.05$)* reduce body weight comparing to normal control. Compared to the negative control, treating with extracts similar to indomethacin significantly reduced paw size and promoted weight gain ($p < 0.05$), whereas the changed inflammatory parameters and MDA was overturned. The data imply that SBCP extracts have anti-arthritic properties equivalent to indomethacin (Aloke et al., 2019).

Table 2.2: Phytochemicals with anti-arthritic activity with mechanism of action

Sr. No	Plant source	Active compound	Chemical structure	Mechanism of action
1	*Acyranthus aspera*	ferulic acid, apigenin		Inhibition of secondary lesions Prevention of the recruitment of leukocytes
2	*Aconitum vilmorinianum Kom.*	Alkaloids and glycosides		Improving swelling, hyperaemia, vascular permeability, and joint allodynia
3	*Bacopa monniera*	Bacosides		Stabilizing action on lysosomal membranes
4	*Cannabis sativum*	delta-9-tetrahydrocannabinol (THC)		Lessened CII-specific proliferation and IFN-g production

5	*Cassia uniflora*	Methyl inositol, Luteolin		Histamine and prostaglandin synthesis inhibition
6	*Clematis chinensis*	Triterpenoid saponins		Inhibited PGE2 production and COX-2 expression
7	*Curcuma longa*	Curcumin		Activation of genes critical to articular inflammation
8	*Glycyrrhiza glabra*	Glycyrrhizin		Lysosomal membrane stability modulation effect, inhibition of leukocyte migration,
9	*Panax ginseng*	Ginsenoside		Suppressed TPA-induced acute inflammation

| 10 | *Sinomenium acutum* | Alkaloids and sterols | | Inhibiting macrophage Function and lymphocyte proliferation, reducing ESR |
| 11 | *Withania somnifera* | Withanolides | | Inhibiting the release of inflammatory mediators |

2.3 Literature review of medicinal plants used in dementia and AD:

Gallantamine and rivastigmine are natural alkaloids that act as acetylcholine esterase inhibitors in the brain, increasing cholinergic firing in the hippocampus and frontal cortex. These medications are heavily promoted and recommended for enhancing cognitive characteristics in Alzheimer's patients. Inhibition of brain cholinesterase activity would result in increased AChE levels and, as a result, increased cholinergic transmission. Inhibition of brain cholinesterase activity would result in increased AChE levels and, as a result, increased cholinergic transmission. In neurodegenerative disorders, herbal candidates that are lipophilic and have antioxidant properties can reduce oxidative and nitrosative stress by increasing reduced glutathione levels, glutathione peroxidase activity, lowering malonaldehyde levels, preventing protein misfolding, and lowering amyloid plaques. Because *clitoriaternatea*, and Brahmi (bacosides) are lipophilic and may cross the blood-brain barrier, they can interact with

endogenous components (Suganthy et al., 2009). Previous clinical trials have found that *M. officinalis* extract decreases laboratory-induced stress and may help with mood improvement (Kennedy et al., 2004).

Polyphenol flavonoids (including rosmarinic acid), monoterpene aldehydes, and monoterpene glycosides are thought to make up the chemical makeup of Melissa and Salvia leaf extracts. *In vitro*, all of these ingredients exhibit a variety of effects, including potent antioxidant action and affinity for muscarinic and nicotinic receptors in cerebral cortex of the human. This final method shows particular relevance since cholinergic system manipulation should help improve cognitive performance, particularly in Alzheimer's disease (Santos-Neto et al., 2006).

Bacosides from the Indian traditional medicinal herb *Bacopa monnieri*, often known as Brahmi, have been shown in several experimental and clinical investigations to improve learning and memory retention. Consumption of a diet high in plant polyphenols reduces cognitive decline and stress in people and animal models, according to research (Joseph et al., 2005; Spencer et al., 2010). A subclass of plant polyphenols, i.e., flavanols with demonstrated pleiotropic activities in neuroprotecting action, are found in a variety of therapies such as cocoa, blueberries, green tea, grapes, and others (Davinelli et al., 2016)**.**, Curcumin from turmeric (*Curcuma longa L.*), Resveratrol from grapes and red wine and epigallocatechin from green tea (*Camellia sinensis*) all have neuroprotective properties (relevance to oxidative methods). Relevant investigation on new extracts of plant and its bioactivities with anti-amnesic effects on diverse neurotransmission systems, mostly from *in vitro* or *in vivo* models, has also been examined (Sun et al., 2008; Frank et al., 2005). In 1996, China produced "Shuangyiping," a tablet formulation of huperzine A for symptomatic

treatment of AD; it is as well sold in United States as a powdered dietary supplement, *H. Serrata* for memory damage (Ramawat et al., 2009).

The most recognized herb for AD is *Ginkgo Biloba*. *Ginkgo biloba* extract exhibited beneficial advantages in controlled clinical studies with a placebo and control group, similarity with prescribed medications as Tactrin or Donepezil, with fewer negative side effects. *Gingko biloba's* main chemical ingredient is gingkolides, which is a potent antioxidant with neuroprotection and cholinergic properties helps in treating AD. *Ginkgo biloba* enhances protective action alongside oxidative damage caused by A proteins (damaging hydrogen peroxide, prevention of lipids from oxidation, and trapped ROS) (Janßen et al., 2010).

In healthy volunteers, the effect of *Salvia rosmarinus* extract on acetylcholinesterase (AChE) action and biomarkers for oxidative stress were studied. AChE activity was considerably reduced after administrating 1000 mg rosemary for 30 days in comparison to AChE action prior to rosemary therapy (*p-value <0.001*) as well in the placebo group (*p-value <0.01*). The findings might support the usage of rosemary as an antioxidant supplement. More investigation should be done to know the usefulness of rosemary in AD patients (Fatemeh et al., 2021).

Ginkgo biloba, a flavanols-rich antioxidant, was investigated for its capacity to counteract aging related behavioural decline and neuropathology in Tg2576 mice. *Ginkgo biloba*-treated mice had higher amounts of protein carbonyls in their brains. In transgenic mouse model of AD, our findings show that prolonged *Ginkgo biloba* administration can prevent an aging-related deterioration in spatial cognition by not affecting A levels or reducing protein oxidation (Stackman et al., 2003).
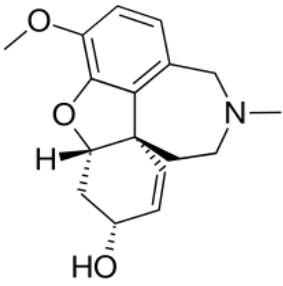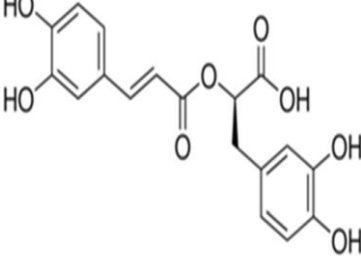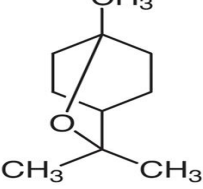
In one of the studies, the leaves of *M. officinalis* extraction with ethanol at a concentration of 80%. Rats were given intraperitoneal injections of *M. officinalis* extract at various dosages (50–400 mg/kg) single or combining with scopolamine (1 mg/kg) before training in Morri's water maze (MWM) over a day. The action of acetylcholinesterase enzyme (AChE) in hippocampus was evaluated after training *M. officinalis* extract could significantly improve naive rats' learning and memory (*p<0.001*) and alleviate scopolamine-induced learning deficits, but dose-dependent effect was not produced, and above 200 mg/kg doses could neither improve naive rats' memory nor reverse scopolamine-induced memory damage. AChE activity was as well inhibited in naive and scopolamine-induced memory-impaired rats. All the findings imply that *M. officinalis* help with memory and also the extract's cholinergic properties might have significance in the memory-improving benefits shown in this study (Soodi et al., 2014).
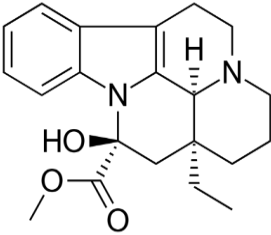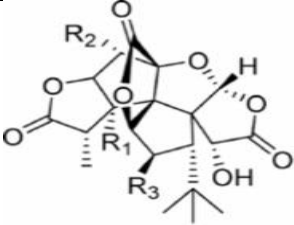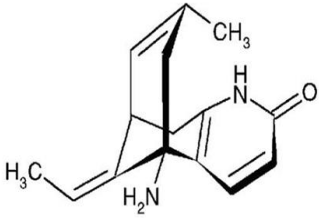
The goal of one of the studies was to see how *Glycyrrhiza glabra* (Gg) root extracts affected learning and memory in male Wistar albino rats about 1-month-old. For six weeks, 4 dosages of aqueous extract of Gg root (75, 150, 225, and 300 mg/kg) were given orally. The findings revealed that all dosages of Gg aqueous root extract considerably improved memory; whereas 150 and 225 mg/kg doses exhibited a significant (*P< 0.01*) improvement in memory and learning. Also, the aqueous root extracts of Gg (150 and 225 mg/kg, p.o.) neurodegenerative illnesses corrected Diazepam-induced amnesia. These data show that Gg's memory-improving properties are mediated through its anti-inflammatory and antioxidant property. As a result, Gg looks as viable medicine for boosting memory in treating learning disabilities, dementia, AD, and various neurodegeneration conditions (Chakravarthi et al, 2013).
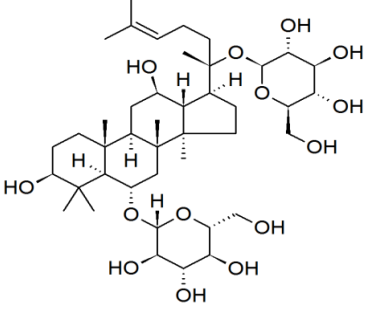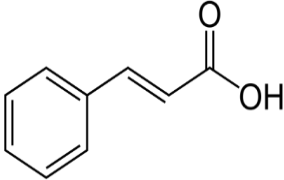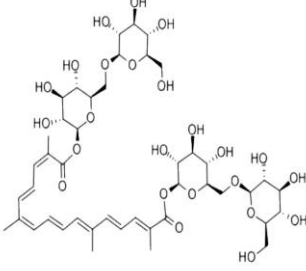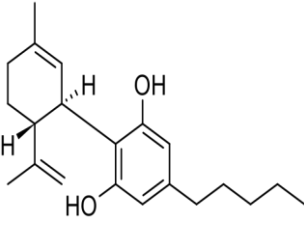
In a 24-week randomised open-label research with Korean red ginseng (KRG), patients with AD demonstrated cognitive advantages. The individuals were recruited to be monitored for two years to establish the lasting effects of KRG. The Korean version of the Mini-Mental Status Examination (K-MMSE) and The Alzheimer's Disease Assessment Scale (ADAS) and were useful to assess cognitive function every 12 weeks, by a daily dosage of 4.5 g or 9.0 g KRG. At 24 weeks shown a considerable progress in the KRG-treated groups. After 24 weeks improving MMSE score stayed unchanged for 48th and 96th weeks in lasting study of the effectivity of KRG. ADAS-cog revealed comparable results. Around week 24, the most significant improvement was discovered. Finally, the impact of KRG on cognitive skills was sustained over two years, showing that lasting follow-up for AD is viable (Heo et al, 2011).
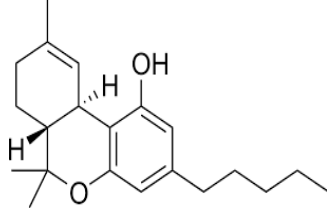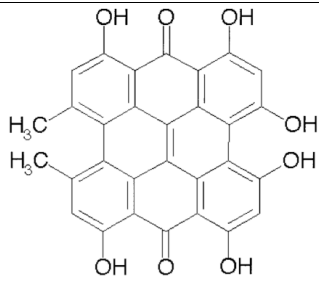
Several Icelandic medicinal plants were studied for their ability to inhibit acetylcholinesterase (AChE). With IC_{50} values of 2.20 mg/ml and 3.56 mg/ml, respectively, ethanolic extracts of *Angelica archangelica* seeds & aerial parts of geranium sylvaticumproved useful. Xanthotoxin and Imperatorin activities from A. archangelica were tested. Xanthotoxin was found to be far more effective than imperatorin, with an IC50 of 155 g/ml (0.72 mm), whereas imperatorin had an IC50 of almost 274 g/ml (1.01 mm). Furanocoumarins, on the other hand, appear to have a modest role in this extract's overall action. The extracts of *A. Archangelica* and *G. sylvaticum* showed a synergistic interaction (Sigurdsson et al., 2007).

Table 2.3: Phytochemicals with anti-dementia activity with mechanism of action

Sr. No	Plant source	Active compound	Chemical structure	Mechanism of action
1	Galanthus species	galantamine		AChE inhibitor also a positive allosteric modulation of nicotinic receptors
2	*Melissa officinalis*	rosmarinic acid		rosmarinic acid with its derivatives seen associating with AChE inhibiting action
3	*Salvia. officinalis* and *S. lavandulifolia*	Monoterpenoids including 1,8-cineole and α-pinene		positive modulators of mood and cognition in healthy younger adults, enhanced secondary memory performance

4	*Vinca minor*	vincamine		Modulating neuronal homeostasis and brain circulation and is antihypoxic
5	*Ginkgo biloba*	EGB761 (flavonoid glycosides and terpenoid)	 Gingkolides A: R₁ = OH; R₂, R₃ = H B: R₁, R₂ = OH; R₃ = H C: R₁, R₂, R₃ = OH	Favourable effects on neuronal cell metabolism, cerebral circulation, cholinergic system; have antioxidant action, reduction in apoptosis, & neuroprotection alongside NO
6	*Huperzia serrata*	huperzines A and B		Improvement in cognitive processes, inhibition of AChE *in vitro* and *in vivo*; higher potency than huperzine B

7	*Panax ginseng*	ginsenosides		Enhances ACh level in CNS, by increase in choline acetyltransferase (ChAT) action or through inhibition of AChE activation
8	*Polygala tenuifolia*	cinnamic acid derivatives and onjisaponins		Inhibit AChE activity in vitro
9	*Crocus sativus*	crocin		Improvement in learning behaviors *in vivo*; crocin reduces TNF-α-induced apoptosis of neuronally differentiated PC12 cells *in vitro*
10	*Cannabis sativa*	Cannabidiol (CBD), 9-tetrahydrocannabinol (THC)		CBD: neuroprotective *in vitro* and antioxidant THC: promotes appetite, as well impairment in

11	*Hypericum perforatum*	hypericin		learning and memory
				show antidepressant activity

Through extensive literature search it has been observed that number of phytochemicals has already been utilised successfully for the treatment of asthma, arthritis and dementia. The phytochemicals are found to be very promising for the management of these diseased conditions without any adverse effects. Considering all these potential applications and benefits of the phytoconstituents we have undertaken the evaluation of Gelsemium sempervirens against dementia, Trigonella foenum-graecum, Piper betel, and Lagenaria siceraria homogenate against arthritis and asthma, Euphorbia tirucalli against arthritis and asthma.

AIM, OBJECTIVES AND PLAN OF WORK

3.1 Aim and objectives of the research work

1. Searching for new and novel medicinal plants which will give promising protection from asthma and arthritis based on scientific literatures and traditional knowledge.

2. To prepare the non-toxic crude herbal extract by cold maceration and followed by sonication in hydro-alcoholic solvent.

3. To screen out all prepared crude extract against lipooxygenase (LOXs) & Cyclooxygenase (COX) whether they are inhibiting or not.

4. To check *in vitro* anti-asthmatic activity in freshly isolated adult goat tracheal preparation.

5. To assay *in vitro* anti-arthritic activity by inhibition of protein denaturation and membrane stabilization model from phyto extract.

6. To assay *in vitro* anti-arthritic activity in proteinase inhibitory action model fromphyto extract.

7. To *in vitro*assay cytokine such as IFN-γ, TNF-α, IL-6, IL-12 and IL-18 from healthy human volunteer donated PBMC cells at post treatment with phyto extract.

8. To check *in vitro* toxicity assay of tested extract on goat lung cells by MTT assay and human RBC lysis assay.

9. To investigate the acute and chronic toxicity study of phyto extract in animal model in order to determine the proper therapeutic dose in *in vivo* studies.

10. To assess *in vivo* anti-asthmatic evaluation in aerosolized-ova-albumin challenged animal model from potential phyto extract obtained from in vitro result.

11. To evaluate *in vivo* anti-asthmatic activity of lead extract in histamine induced broncho constriction model in guineapig tracheal chain preparation model.

12. To evaluate *in vivo* anti-asthmatic activity by histological analysis of lung tissues in asthma model.

13. To evaluate *in vivo* anti arthritic activity in Complete Freund's Adjuvant (CFA) induced poly-arthritic rat model by measuring paw volume and ankle diameter.

14. To investigate the delayed type hypersensitivity response (DTH) assay in chronic inflammation rat model to measure the protection level by the phyto-extract.

15. To check the *in-vitro* inhibition study of rat brain homogenate enzymes acetylcholine-esterase (AchE), β-secretase (BACE), by individually separatedfractions, for lead drug development against neurodegenerative disorders.

16. To check the cerebro-protective effects of respective three lead *phyto-* fractions and isolated phytomarkers against *in vitro* brain homogenate lipid peroxidation & assessment of *in vitro* anti-oxidant free radical scavenger activity to understand the mechanism of neuroprotective activity.

17. To evaluate the anti-dementia & cerebroprotective against: Cognitive impairment in a mice model of scopolamine induced amnesia; Passive avoidance response & elevated plus maze.

18. To perform preliminary screening of several plant fractions, to see whether the experimental drug candidates are inhibiting *in vitro* cytokine release in human PBMC cell culture after LPS stimulation.

19. To evaluate the *in-vitro* doses of test compounds (at varied concentration) & to determine the IC_{50} values.

20. To formulate a cheap therapeutically active & safe poly-herbal medicine for the rural population in India suffering from arthritic inflammatory disorders.

Proposed work flow diagram (Dementia)

3 respective bioactive lead fractions by preparative HPLC, TLC followed by column chromatography.

In-vitro **inhibition screening of brain homogenate supernatant & kits containing:**
a) **AchE**
b) **BACE**
c) **TBARS (lipid peroxidation)**

Again *in vitro* **screening of isolated compounds upon initial enzyme targets**.

Qualitative characterization of preferred compound with help of standard in HPTLC, HPLC.

Selection of lead fractions based on inhibitory results.

TLC, column, HPTLC, preparative HPLC of selected fractions

Selected compound dose selection by haemolysis asay, MTT assay, LD50 values.

Formulation of isolated phytomolecules& fractions.

In-vivo dementia study
1. Scopolamine induced dementia model.
Learning & Memory evaluation in: **(a) Passive avoidance response, (b) Elevated Plus Maze(c) Morris water maze (d) Radial arm maze.**

Only significant & positive outcome oriented study group would be sacrificed.

Mice brain isolation & estimation of biochemical parameters: AChE activity, MDA, GSH, Nitrite levels, neurotransmitters levels by HPLC.

Long term toxicity tests: nephrotoxicity, hepatotoxicity, body weight, complete blood count, Heamolysis assay.

Proposed work flow diagram (arthritis and asthma)

Evaluation of *Trigonella foenum-graecum*, *Piper betel*, and *Lagenaria siceraria* homogenate against arthritis and asthma

```
┌─────────────────────────────────────────────┐
│   Extraction of Trigonella foenum-graecum,    │
│      Piper betel and Lagenaria siceraria.     │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│          Phytochemical screening:             │
│  Saponin, Proteins and amino acids,           │
│  flavonoids, Glycosides, Lignin, Mucilages,   │
│  Polyphenol and total solids                  │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│   In-vitro anti oxidant activity, protein     │
│  denaturation assay, anti-proteinase activity,│
│      membrane stabilization study, 15-        │
│       lipoxygenase (LOX) inhibition study     │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│                  In-vitro                     │
│       Anti-asthmatic activity (bioassay)      │
│        Pro-inflammatory cytokine assay        │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│      In vivo CFA induced anti-arthritic       │
│        appraisal  study in a rat model        │
└─────────────────────────────────────────────┘
```

Proposed work flow diagram (arthritis and asthma)

Evaluation of *Euphorbia tirucalli* against arthritis and asthma

Extraction and preparation of steroid–terpenoid
rich fraction from *Euphorbia tirucalli*

↓

Phytochemical test and thin layer
chromatographic study of STF-HAETR

↓

Phytochemical characterization using HPTLC

↓

In vitro pro-inflammatory cytokine and nitric
oxide evaluation

↓

Assessment of pro-inflammatory mediators' iNOS
by
western blot analysis

↓

In-vitro cyclooxygenase (COX) inhibition
appraisal of STF-HAETR:

↓

In vitro
anti-asthmatic evaluation
inhibition of protein denaturation
Proteinase inhibitory action
Membrane stabilization studies

↓

In vivo assessment of anti-nociceptive pain of
STFHAETR
Anti-nociceptive pain assessment by the hot-plate
technique

↓

In vivo anti-inflammatory evaluation and acute
toxicity study

I. Evaluation of *Gelsemium sempervirens* against Dementia

Chemicals, Compounds, and Drugs source:

Table 4. 1: List of chemicals, compounds, and drugs with source

SR. No	Chemicals /Compounds/Drugs	Source
1	Mother-tincture of *Gelsemium sempervirens* (57-61% alcohol)	SBL Laboratories Ltd., Uttarakhand, India
2	Piracetam (98%; HPLC grade	Sigma
3	Scopolamine hydrochloride (HPLC grade, 90%	Sigma-Aldrich
4	Galanthamine hydrobromide (TLC grade, 94% pure)	Sigma-Aldrich

Experimental animals:

In this study, male Swiss albino mice (25-30 gm weight), and 4-week-old were used. For all animal experiments prior approval of animal ethical committee was taken and experimental procedures were conducted as per the guidelines The mice were housed in polyacrylic cages (38 X 23 X10 cm^3) with five individuals per cage and were kept in typical laboratory settings (Rt 24–27°C and RH 60–65%). The animals were provided with food and water was freely accessible. Five days before the experiment, the mice were acclimated to the laboratory environment. All animal studies followed the National Institutes of Health's Guidelines for the Care and Use of Laboratory Animals. The Pharmacologic procedures were authorised by the Institutional Animal Ethical Committee of Dr. B. C. Roy College of Pharmacy and A.H.S., which was constituted

under the Committee for the Control and Management of Experiments on Animals.

Acute oral toxicity studies:

The acute oral toxicity study was performed as per Organization for Economic Co-operation and Development (OECD) test guidelines (Test no., 425, 2008) following the Up-and-Down procedure.

Up-and-Down procedure evaluates LD50 value with a confidence interval for assessing acute toxicity according to the Globally Harmonised System of classification and labelling of chemicals. The test drug is usually given to animals who have fasted prior to dosing in a single dosage via gavage. Dosing is normally done in a sequential way at 48-hour intervals, with the first animal dosed a step below the best preliminary LD50 values. Subsequently, lower dose is given to the second animal in case the first dosed animal doesn't survive or high dose is recommended if the first dosed animal well survived. All the animals are kept under observation for the period of 14 days in which a special attention was given for initial 4 hours The LD50 was estimated by followed by computation of respective interval estimates for the LD50 can also be done.

Experimental procedure:

The phytochemical composition of procured tincture was well characterized and manufactured as per Indian homeopathic pharmacopeia. Appendix – XXXIII and French homeopathic pharmacopeia, 2002, ANSM general monographs. It was used as reconstituted lyophilized powder-residue redissolving in water for injection (WFI) immediately to study the anti-dementia activity at a dose of 1 mg/kg orally daily for consecutive 14 days. The dose was selected based on 1/10th of LD50 doses, carried out as per OECD and WHO guidelines. All experimental animals were divided in to 5 groups with 6 mice in each of the group for the evaluation of cognitive measurements in

respective exteroceptive behavioral models. Treatment protocols are described schematically in the following table 4. 2

Table 4.2: Treatment protocol followed during the study

Group	I vehicle control	II untreated control	III, *Gelsemium* treated group (without scopolamine)	IV *Gelsemium* treated group (with scopolamine)	V positive control (standard group)
No. of mice	6	6	6	6	6
Treatment	WFI orally for 14 days	WFI orally for 14 Days þscopolamine. after 60 min of the last dose of WFI	Reconstituted and lyophilized *Gelsemium* in WFI 1 mg/kg orally for 14 days	Reconstituted & lyophilized *Gelsemium* powderdissolved in WFI 1 mg/kg orally for 14 daysþscopolamine 1 mg/ 60 min of the last dose of	Piracetam 200 mg/kg IP from 8th to 14th dayþscopolamine1 mg/kg IP on 14th day after 60 min of the last dose of piracetam
Procedure	Behavioral test performed after 90 min of last WFI dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant	Behavioral test performed after 30 min of scopolamine dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant	Behavioral test performed after 90 min of last *Gelsemium* dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant	*Gelsemium* Behavioral test performed after 30 min of scopolamine dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant	Behavioral test performed after 30 min of scopolamine dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant

Cognitive measurements

Elevated plus maze (EPM) test:

This model was developed as per the procedure described in the literature with slight modifications (Kulkarni et al., 2011). The instrument used in this study consisted of two open arms (16 cm X 5 cm) and two covered arms (16 cm X 5 cm X 12 cm). A maze was raised to a height of 25 cm from the floor by extending the arms from a central platform (5 cm X 5 cm). On the first day, each mouse was placed at the end of an open arm facing away from the centre platform. Transfer latency (TL) was assessed as the time it took the mouse to go inside any of the covered arms with all four legs. TL was recorded on

the first day (day 14). If the animal did not enter one of the two covered arms within 90 seconds, it was gently pushed into one of the two covered arms with the TL set to 90 seconds. Before being returned to its home cage, the mouse was given 10 seconds to explore the maze. The next day (day 15), memory recall was tested by recording the TL once more.

Passive shock avoidance paradigm:

To investigate long-term memory, this test based on negative reinforcement was conducted (Jeong et al., 2008). The apparatus used in this study had dimensions of 27x 27 x 27 cm^3 with 3 wooden walls and one Plexiglas wall, a grid floor and a wooden platform (10 x 7x 1.7 cm3) at the grid floor's middle. During the experiment, a 15 W bulb was used to light the enclosure. The grid floor received a 20 V AC electric shock. Two comparable sessions of training were held. Each animal was carefully put on the wooden platform. During the experiment, a 15 W bulb was used to light the enclosure. The grid floor received a 20 V AC electric shock. Two comparable sessions of training were held. Each mouse was carefully put on the wooden platform in the grid's middle. Shocks were provided for 15 seconds after the mouse stepped down and placed all of its paws on the grid floor, and the step-down latency (SDL) was measured. The time it took the mouse to step down from the wooden platform to the grid floor with its full paw was defined as SDL. During the first test, animals with SDL in the range (2–15 s) were used for the second session and retention test. The second session took place 90 minutes after the first. Electric shocks were provided for 15 seconds if the animals stepped down before 60 seconds. If the animals did not step down for 60 seconds during the second test, they were removed from the shock-free zone. After 24 hours, retention was evaluated similarly, with the exception that no electric shocks were administered to the

grid floor. Each mouse was put on the platform twice, and the SDL was recorded with a 300-second higher cut off time.

Estimation of biochemical parameters:

All the biochemical parameters were estimated at the end of the behavioral test. The details of all the tests are mentioned below.

Brain tissue preparation:

After the behavioural investigations were completed, the brains of treated and untreated mice dissected followed by washing with cold saline solution in a petri plate maintained on ice. Tissues were weighed and homogenised at 9500 rpm in an ultra turrax T-25 homogenizer with 0.03 M pH 7.4 phosphate buffer to produce a 10% w/v homogenate that was utilised in subsequent calculations. Similarly, entire brain homogenates from normal healthy mice were produced as described above to measure *in-vitro* acetylcholine esterase activity.

Protein estimation from brain samples:

Bradford's reagent (0.01-0.1 mg/ml range) was used to assess protein in all supernatants of brain samples, using BSA as a reference (Bradford., 1976).

***In-vitro* brain acetylcholinesterase (AChE) activity estimation:**

In a Beckman Ultracentrifuge, the brain homogenate was centrifuged for 60 minutes at 100,000g at 41^0 C. The supernatant solution so obtained was collected and kept at 41^0 C for measurement of acetylcholinesterase using Ellman's technique with minor changes (Ellman et al., 1961). *In vitro*, the impact of Gelsemium sempervirens crude hydro-alcoholic tincture on the activity of supernatant containing AChE was investigated at various doses (2–75 mg/ml) with a final reaction volume of 3.14 ml. 0.4 ml aliquots of the supernatant (source of AChE enzyme) were incubated with 20 ml of aliquots of

tincture for 5 min at 37^0 C and put to a cuvette with 2.6 ml phosphate buffer (0.1 M, pH 8) and 100 µl of 5,50-dithiobis (2-nitrobenzoic acid (DTNB (98% pure, as the TLC grade from Sigma-Aldrich). The reaction was followed by addition of 20 µl acetylthiocholineiodide ((ATCI; 99 percent pure, as the TLC grade from Sigma-Aldrich)), an AChE enzyme substrate. The 2-nitro-5-sulfidobenzene-carboxylate anion (yellow colour) generated by the interaction between DTNB and thiocholine released by the enzymatic hydrolysis of acetylthiocholineiodide for 10 minutes was detected spectrophotometrically at a wavelength of 412 nm. The % cholinesterase inhibitory activity was estimated by following equation (Ahmed et al., 2010).

Inhibition of AChE (%) = (δ A control - δ A sample/ δ A control)/ δ A control X 100

In vitro **BACE1 (β-secretase) enzyme assay:**

This assay was performed with slight modifications as per the procedure described by Jeon et al., 2003. A combination of 10 ml of assay buffer (50 mM sodium acetate, pH 4.5), 10 ml of BACE1 (1.0 U/ml; Sigma-Aldrich), 10 ml of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate (Sigma)), and 10 ml of the reconstituted mother tincture (2–75 mg/ml) diluted in 0.2 percent Each sample's fluorescence was measured at 540 nm for the exciting wavelength and 580 nm for the emission intensity. Following formula was used to estimate the % inhibition

Inhibition % = [1 - {(S S$_0$) / (C C$_0$)}] X 100

where

C = fluorescence of the untreated control (enzyme, buffer, and substrate containing 2% DMSO) after 60 min of incubation,

C_0 = the fluorescence of control at zero-time, S is the fluorescence of the tested samples (enzyme, sample solution, and substrate) after incubation,

S_0 = fluorescence of the tested samples at zero time.

The IC50 value was estimated using Graph Pad Prism 4.0 (Graph Pad Software Inc., USA) through regression analysis.

Estimation of brain acetylcholinesterase activity *in vivo:*

In a cuvette containing 2.6 ml phosphate buffer (0.1 M, pH 8) and 100 l of DTNB, drug-treated and untreated supernatant (0.4 ml aliquot) samples obtained from brain homogenate were added. The absorbance was measured at 412 nm using A UV–visible spectrophotometer after the contents of the cuvette were completely mixed (Shimadzu, USA). When the absorbance attained a steady value, it was recorded as the baseline measurement followed by the addition of 20 l acetylthiocholine iodide as substrate. For a total of 10 minutes, the change in absorbance was measured every 30 seconds. The amount of micromoles of acetylthiocholine iodide hydrolyzed per minute per mg of protein was used to define one unit of acetylcholinesterase activity (Srikumar et al., 2004).

Estimation of brain reduced glutathione (GSH) levels

When GSH reacts with DTNB, it creates a yellow chromophore that can be identified by spectrophotometry. GSH was estimated using a brain homogenate combined with an equivalent quantity of 10% TCA and centrifuged at 2000g for 10 minutes at 4 1C. In 0.1 mol of the processed tissue sample, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of DTNB, and 0.4 ml of double-distilled water were added, and the mixture was forcefully agitated on vortex. The UV–visible spectrophotometer was used to measure the absorbance at

412 nm (Shimadzu, USA). GSH was calculated using a reduced glutathione standard curve and represented as mg/mg protein (Tota et al., 2011).

TLC bioautographic assay with reconstituted Gelsemium tincture through true TLC based Ellmans's method following thiocholine and DTNB based inhibition reaction:

Gelsemium tincture was reconstituted and diluted in methanol and water (1:1 v/v) to make a 20 g/ml solution. The mobile phase consisting chloroform: methanol (9:1 v/v) and 2 %, antimony trichloride in 10% methanolic KOH as spray reagent, a drug-loaded TLC plate (stationary phase: silica gel) was produced for chromatogram. Spraying 5 mm ATCI, DTNB (dissolved in 50 mM Tris HCl buffer, pH 8 and 37^0 C), and drying the plate thoroughly dried and saturated it. Then, according to Ellman's approach, 3 U/mol of AChE (50 mM Tris HCl buffer, pH 8, 37 1C) was sprayed on the plate, resulting in the yellow-coloured background with white dots, which are AChE inhibiting extracts measured after 5 minutes. The false-positive reactions were identified using the method previously described to determine if the positive outcomes obtained in the TLC or microplate assays by the reconstituted Gelsemium tincture were due to true enzyme inhibition or inhibition of the chemical reaction between DTNB and thiocholine. Another plate was produced and sprayed with 5 mm of DTNB (50 mM Tris HCl buffer, pH 8, 37 1C), then sprayed with 5 mm of ATCI and 3 U/mL of AChE dissolved in 50 mM Tris–HCl buffer, pH 8, the appearance of white specks on a yellow background mimic false-positive finding. Galanthamine (10 mg/ml) was utilised as a positive control in both cases of the experiment, along with the Gelsemium extracts that were examined. In both cases, white specks on a yellow backdrop were captured and compared to each other. The findings obtained with TLC assay were compared with results of the

spectrophotometric evaluation were compared to the results of the TLC assay (Rhee et al., 2003).

Acetylcholinesterase mRNA expression by reverse transcription PCR-:

The extraction of RNA was performed form the treated and untreated brains of the mice using TRIzol reagent (Sigma). Using Gene Quant, the concentration and purity of RNA were measured spectrophotometrically. In a 20-ml combination including oligo-(dT)-primer, RNase inhibitor, dNTP mix, and 5X reaction buffer, approximately 2 mg of total RNA was reverse transcribed using reverse transcriptase (RT) (Omniscript RT kit). Using the Taq PCR core Kit, the resulting cDNA was amplified independently with specific primers for AChE. (Qiagen USA). cDNA was amplified with specific primers in a 20 ml reaction volume containing 1 U Taq polymerase, 200 mM (each) dNTP mix, and 2 ml 10X Taq buffer. The polymerase chain reaction mixture was amplified for 35 cycles in a DNA thermal cycler according to the following specifications:

Primer AChE 50-GATCCCTCGCTGAACTACACC-30 Annealing temperature 60^0C. 50-GGTTCTTCCAGTGCACCATGTAGGAG-30, Product size 331 bp (Tota et al., 2011).

Statistical analysis:

The results obtained in this study were expressed as mean of 7 std error. The statistical analysis was performed with unpaired students t-test, ANOVA and Turkey's posthoc analysis. The results with P<0.05 were considered as statistically significant.

II. Evaluation of *Trigonella foenum-graecum*, *Piper betel*, and *Lagenaria siceraria* homogenate against arthritis and asthma

Chemicals, Compounds, and Drugs source:

Table 4.3: List of chemicals, compounds, and drugs with source

SR. No	Chemicals /Compounds/Drugs	Source
1	Sealed pack methi (Fenugreek seeds) (*Trigonella foenum-graecum*).	Big bazaar supermarket, Durgapur, West Bengal, India
2	Betel leaf (*Piper betel)*	Local market at IIT, Kharagpur, West Bengal, India
3	Fresh, green, and edible variety of bottle gourd (*Lagenaria siceraria*)	Local market at IIT Kharagpur
4	Lipo-poly saccharide (LPS)	Sigma-Aldrich
5	Bovine serum albumin (BSA; Catalogue No A9418, Sigma; purity ≥ 96%),	Sigma-Aldrich
6	α- chymotrypsin	Sigma-Aldrich
7	β-casein (C6905-1G; ≥ 98%),	Sigma-Aldrich
8	Freund's complete adjuvant (CFA)	Sigma-Aldrich
9	2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich
10	Hydrocortisone ((≥98%),	Sigma-Aldrich
11	Aspirin (≥99%),	Sigma-Aldrich
12	Pure cucurbitacin B	Sigma-Aldrich
13	Epigallocatechin gallate (≥97%)	Sigma-Aldrich
14	Azelastine solution	Renuka Medical (Pharmacy shop), Kolkata, India
15	Gallic acid	Loba Chemie Pvt. Ltd. Mumbai, India.
16	Ruthenium red	Loba Chemie Pvt. Ltd. Mumbai, India.
17	Absolute ethanol	Honyon International, Inc., China
18	Acetonitrile	Sigma-Aldrich
19	HPLC grade water	Sigma-Aldrich

Experimental animals:

In this study female albino Wistar rats (150-230 gm weight) were used for in vivo pharmacological screenings. For all animal experiments prior approval of animal ethical committee was taken and experimental procedures were conducted as per the guidelines

Extraction of *Trigonella foenum-graecum*, *Piper betel* and *Lagenaria siceraria:*

The peel was gently removed from the delicious bottle gourd fruit that had been purchased from the market. A commercial juicer (Prestige, Kolkata, India) was used to extract juice from 500 gm bottle gourd fruit. Because extracted juice includes a lot of suspended particulates, it was cloth filtered before being used in tests.

Fresh Bangla variety Piper betel leaves were collected from a local market of IIT Kharagpur and dried in air. The dried leaves were weighed after being cut into pieces. 80 g of leaves were put in the beaker containing water, heated to 60°C in a water bath with a leaf to water ratio of 0.8 for 60 minutes, and filtered using a cloth filter to get the piper leaf extract.

Fenugreek seeds (200 gm) were added to distilled water maintaining a seed to water ratio of 0.2. The solution was warmed at 40°C for 60 minutes to ensure the complete extraction of bioactive compounds.

Phytochemical screening:

The obtained extracts in the above step were screened for various phytochemicals which are described below

Saponin test:

The test was performed with dilution of extract with distilled water (20 ml) for 15 minutes followed by shaking in measuring cylinder. The saponins were confirmed with observation of foam of 1 cm layer (Bhandary et al., 2012).

Proteins and amino acids:

a) Xanthoproteic Test:

A few drops of concentrated nitric acid were added to the extracts. The presence of proteins was confirmed by the development of a yellow colour.

b) Ninhydrin Test:

Few drops of ninhydrin reagent (0.25% w/v) was added to the extract followed by boiling for specific time. The presence of amino acid was detected by the formation of blue colour (Bhandary et al., 2012).

Detection of flavonoids:

The extracts were treated with a few drops of sodium hydroxide solution. When dilute acid was added, the presence of flavonoids was recognised by the production of a bright yellow colour that became colourless (Bhandary et al., 2012).

Test for glycosides:

5 mL of the sample was taken into two separate test tubes. One had hydrochloric acid added to it. Both the samples were boiled for some time after adding Fehling's solution A and B. The appearance of a red brick precipitate with greater intensity compared to HCl untreated test tube in the hydrochloric acid test tube showed the presence of glycosides (Bhandary et al., 2012).

Test for Lignan:

In test tubes (10 ml each), small samples of the extract and homogenate were taken, and gallic acid was added to each sample (50 mg). The presence of lignan was shown by the formation of an olive-green colour (Mounguengui et al., 2016).

Test for mucilages:

The presence of mucilage in the aqueous extract and bottle gourd homogenate was determined using the Ruthenium red test (Priya et al., 2011). A small amount of ruthenium red dye was added to the samples after they were diluted with water in a 1:5 ratio (5 mg). The presence of mucilages was indicated by the appearance of pink colour.

HPLC study of experimental test extract:

Piper betel, fenugreek, and bottle gourd aqueous extracts were injected in different modes using C-18 reverse phase HPLC column (4.6 mm I.D., 250 mm length, and 5 mm particle size), The mobile phase for piper betel was 0.1% orthophosphoric acid (H_3PO_4) and 100% acetonitrile at a flow rate of 1 ml/min, with a detection wavelength of 200 nm. Pure cucurbitacin B and bottle gourd extract was also subjected to HPLC analysis as per the procedure described (Krepsky et al., 2009). The analysis was performed under isocratic mode with flow rate of 1.2 ml/min, and acetonitrile: water in a 40:60 ratio as mobile phase. Under isocratic elution, the detection was done at 230 nm. Water, acetonitrile, and acetic acid made up 94.9/5/0.1 % of the mobile phase. The absorbance was fixed at 245 nm and the flow rate was set at 1 ml/min (Joshi et al., 2014).

 Polyphenol estimation:

A modified Folin and Ciocalteu technique was used to determine total polyphenol. with the aid of a spectrophotometer (Perkin Elmer, Shelton, CT, USA). The findings were measured in mg of Gallic acid equivalent (GAE) per 100 milliliters of water (Karmakar et al., 2017).

Total solids:

The total solid content from the sample was determined by heating of the extract at 104±2°C in hot air oven till the weight difference in the juice remained constant at repeated intervals (Karmakar et al., 2017).

Antioxidant activity:

The antioxidant activity was determined by DPPH assay in each extract

0.5 mL sample, 3 mL absolute ethanol, and 0.3 mL DPPH radical solution made up the reaction mixture. When DPPH combines with an antioxidant molecule, the deep violet colour turns to pale yellow. After 100 minutes of treatment, the absorbance of the sample was determined at 517 nm using UV-vis spectroscopy. By combining the sample (0.5 ml) and the DPPH radical solution (0.3 ml) with ethanol, the blank and control were created (Mensor et al., 2001). The antioxidant potential was determined using following equation

$$AA\% = 100 - \left[\frac{(Abs_{sample} - Abs_{blank}) \times 100}{Abs_{control}} \right]$$

***In vitro* protein denaturation assay:**

To test the protein denaturation experiment, a 5 wt % Bovine Serum Albumin aqueous solution was prepared in Milli-Q grade water and 98 µL was added to each well of falcon 96 well microplates. Then, in each well, µL of each test extract (fenugreek, bottle gourd, and betel leaf) were added in graded concentrations (total solids) ranging from 50 to 200 g/ml. For 25 minutes, the plate was incubated at 72° C. After freezing the samples on ice for 5 minutes, 100 µL saline phosphate buffer of pH 6.3 was added to each well to prevent further denaturation. At 660 nm, the absorbance of turbidity as an end was measured in a Micro-plate Reader. Hydrocortisone was used as a positive control for the

denaturation of BSA (Palit et al., 2016). The percentage inhibition of BSA denaturation was determined using following equation

$$\% \text{ inhibition} = \frac{\text{absorbance of blank- (absorbance of sample-absorbance of sample background)}}{\text{absorbance of blank}} \times 100$$

In vitro **anti-proteinase activity:**

The anti-tryptic potential of bioactive extract was measured to analyse *in vitro* anti-proteinase activity in an arthritic model. The final reaction mixture (1 ml) contained 200 µL chymotrypsin solution in 20 mM tris-buffer (pH 7.4) at 60 g/ml concentration, 2 µL test extract of fenugreek, betel leaf, and bottle-gourd at varied concentrations of total solid (50-200g/l), and 198 L double distilled (DD) water. After 5 min of incubation at 37°C, 200 µL of 0.8 percent (w/v) casein solution was added to each reaction mixture in an Eppendorf tube. Each reaction tube was then incubated at 37°C for another 20 minutes before being centrifuged at 7500 rpm for 5 minutes following the addition of 400 µL 70% perchloric acid to stop the enzymatic process. Finally, the supernatant from each tube was carefully removed followed by measurement of absorbance at 280 nm by spectrophotometrically. This study employed hydrocortisone, a proteinase inhibitor, as a positive standard control. The blank was made up of trypsin and casein in double-distilled water in this case. It should be emphasised that the % inhibition of proteinase activity was calculated in comparison to chymotrypsin activity (Palit et al., 2016). The following equation was used to compute the percentage inhibition of proteinase activity in the test extracts.

$$\% \text{ inhibition} = \frac{\text{absorbance of blank- (absorbance of sample-absorbance of sample background)}}{\text{absorbance of blank}} \times 100$$

Effect of extract on membrane stabilization:

The membrane of the human red blood cell (HRBC) must be stabilised via hypotonicity-induced membrane lysis.2 ml hypotonic saline (0.25 percent NaCl), 1 ml 0.15 M phosphate buffer (pH 7.4), and 1 ml test extract of fenugreek, betel leaf, and bottle-gourd (50, 100, and 200 g/ml of final volume) in normal saline, with 0.5 ml HRBC suspension (10% v/v) made up the 4.5 ml assay combination. Instead of test solution, 1 mL of isotonic saline was used in control testing, and product control tests did not include red blood cells. For 30 minutes, the mixtures were incubated at 56° C. The tubes were chilled for 20 minutes under running tap water. After centrifuging the mixtures, the absorbance of the supernatant was measured at 560 nm (Palit et al., 2016). Percentage membrane stabilizing activity was determined using following equation:

$$\% \text{ Stabilization} = 100 - \frac{(\text{absorbance of sample} - \text{absorbance of control}) \times 100}{\text{absorbance of control}}$$

The control represents 100% lysis.

***In vitro* 15-lipoxygenase (LOX) inhibition study by test extract:**

Lipoxygenase Inhibitor Screening Assay Kit (760700, USA) was utilised to explore the lox inhibitory studies of bio-active water extract of bottle gourd and betel leaf. The enzyme inhibition investigation was performed as per manual. To conduct this investigation, test extracts ranging from 10, 50, and 100 g/ml were used. In this investigation, a similar dose of azelastine, a common lox inhibitor, was used as a positive control. Using linoleic acid as the 15-LOX substrate, final absorbance was measured at 490 nm in a Microplate Absorbance Reader through a 96 well plate.

Percentage inhibition was estimated as per the following formula.

$$\% \; inhibition = \dfrac{\left[IA - inhibitor\right]}{IA} \times 100$$

Where IA is 100% initial activity.

In-vitro **anti-asthmatic activity (bioassay):**

Adult goat trachea that had been freshly extracted was taken from a local slaughterhouse and prepared for testing. Under continuous aeration at 37°C, the tracheal chain was suspended in an organ bath containing Kreb's solution, which contained sodium chloride 6.9 g/l, potassium chloride 0.35 g/l, calcium chloride 0.28 g/l, magnesium sulphate 0.28 g/l, sodium bicarbonate 2.1 g/l, potassium dihydrogen phosphate 0.16 g/l, and glucose 2.0 g/l. By maintaining a 5-minute time cycle, a dosage response curve for histamine was constructed in various concentrations. Following the acquisition of a histamine dosage response curve on the trachea, 300 g/ml of bottle gourd aqueous extract was added to the appropriate reservoir, along with a 50 g/ml histamine dose (Dhonde et al., 2008).

In vitro **pro-inflammatory cytokine assay in the supernatants by ELISA method**

From cultured peripheral blood mononuclear cells (PBMCs):

Histopaque-1077 (Sigma, Israel) gradient centrifugation was used to separate peripheral blood mononuclear cells (PBMCs) from the venous blood of healthy volunteers. The cells were washed and grown using RPMI-1640 medid containing 1% penicillin, streptomycin, nystatin, and 10% foetal calf serum, according to the protocol (FCS). In 1 ml of complete medium, 2 x 10^6 PBMCs were suspended. The cells were treated for 24 hours with 20 ng/ml lipopolysaccharide to induce tumor necrosis factor (TNF-∞) and interleukin-6 (IL-6) production (LPS; Lipopoplysacharide W E. coli 055: B5, Sigma). After 24 hours of incubation with LPS, 200 g/ml of fenugreek, bottle gourd, and betel leaf extract were added. The cells were extracted by centrifugation at 1500 rpm for 10

minutes after the incubation period. The supernatants were withdrawn. The inflammatory cytokine content of IL-6 and TNF-α was measured using an ELISA kit according to the manufacturer's instructions (e-bioscience, California, USA).

***In vivo* CFA induced anti-arthritic appraisal in a rat model:**

All of the animals were separated into seven groups, each with six animals. Group I was given DD water as vehicle control, group II was given untreated control (CFA, 100 l), and groups III-VI were given BLWE (betel leaf water extract), FGWE (fenugreek water extract) (CFA, 100 μl extract) at doses of 100 mg/kg and 200 mg/kg, respectively. The dexamethasone injection i.p. at a dosage level of 0.75 mg/kg was used as a positive control standard in Group VII. To cause arthritis, 100 μl of CFA (containing 10 mg/ml of heat-killed Mycobacterium tuberculosis in paraffin oil) was injected into the sub plantar area of each rat's right hind paw. Day 1 was defined as the day of the CFA injection. The oral delivery of the BLWE, FGWE, and dexamethasone injection, as well as the vehicle (0.02 (M) PBS) to all groups, began on day 14 and continued until day 28. On days 0, 4, 8, 12, 16, 20, 24, and 28, the anti-arthritic potential of BLWE and FGWE was assessed using paw volume edema. Each paw was also ranked on an ordinal scale, as follows: 0 = unaffected, 1 = one type of joint, 2 = two types of joints, 3 = three types of joints, 4 = four types of joints, with maximum erythema and swelling. The maximum arthritic score per rat was set at 4 (4 points plus 1 hind paw). All experimental groups were assessed on the 15th, 21st, and 28th days after the start of the study (Kokkola et al., 2003).

III. Evaluation of *Euphorbia tirucalli* against arthritis and asthma

Chemicals, Compounds, and Drugs source:

Table 4.4: List of chemicals, compounds, and drugs with source

SR. No	Chemicals /Compounds/Drugs	Source
1	The root of *Euphorbia tirucalli Linn* along with the whole plant	The local area of Durgapur
2	70% ethanol	Sigma Aldrich, India
3	N-hexane	Sigma Aldrich, India
4	Acetone	Sigma Aldrich, India
5	Benzene	Merck, India
6	Ethyl acetate	Merck, India
7	Formic acid	Merck, India
8	RPMI-1640 medium	Sigma, India
9	DMSO	Sigma, India

Extraction and preparation of steroid–terpenoid rich fraction from *Euphorbia tirucalli* Root:

For hydro-alcoholic extraction by cold maceration, 1200 g (Wi) air-dried root powder of *Euphorbia tirucalli* was utilised. The dried powder was placed in a 5 lit conical flask, which was then filled with 4 liters of 70% ethanol solution. Following a thorough soaking of the dry powder in the extracting solvent, the menstruum was shaken continuously for 3 hours before being stored at 4 degrees Celsius for 15 days. Every three days, the solution was shaken with an agitator to adequately disseminate the phytoconstituents into the solvent. The hydroethanolic extract was transferred to a new container after 15 days. Re-maceration with new solvents was used to extract remaining phytocompounds from marc (residual cellular debris accessible after extraction from

organized dried powdered stem). The entire solution was already filtered. To produce dry crude drug residue (Wf) including phyto-extract, the filtrates were evaporated to dryness using a rotary vacuum evaporator. Finally, the extractive value yield percent (We) was computed. The extract was partitioned using n-hexane and ethyl-acetate solvents, yielding two 75 and 50 g fractions, respectively. For silica gel column chromatography, an N-hexane fraction was used, which was then eluted with hexane–acetone (95:5–5:95) to provide 10 fractions (A–J). TLC and phytochemical tests were performed to confirm the presence of steroid and terpenoid in those fractions. Finally, the fractions were mixed and evaporated to dryness in a rotating vacuum to provide a dry residue of 13.6 g of total steroid and terpenoid rich fractions of E. tirucalli root hydro-alcoholic extract, which was coded as (STF-HAETR) and validated by phytochemical tests and TLC.

Phytochemical test and thin layer chromatographic study of STF-HAETR:

The existence of phytochemical groups such as alkaloids, steroids, flavonoids, terpenoids, glycosides, saponin, and tannins on extracted crude phyto-extract was investigated According to (Paech et al., 1955). TLC was used to create a chromatogram in the mobile phase in chloroform: methanol (9:1) and chloroform: ethyl acetate: methanol in both crude and STF-HAETR extracts (2:4:4) (Harborne 1998). To describe the extract, the Rf value was computed from the generated chromatogram. Different chemical spraying reagents were sprayed into the TLC plate to identify the specific phytochemical group and find their location in the produced chromatogram. Two chemical spraying reagents, vanillin–phosphoric and p-anisaldehyde sulfuric acid, were sprayed to the TLC plate to identify the particular phytochemical group and pinpoint their location in the produced chromatogram. The presence of phytochemical groups

was established by altering the band colour at their matching Rf after spraying the reagent was treated. In the same solvent solution, the chromatogram of STF-HAETR was compared to the conventional phytomarkers Lupeol and b-sitosterol.

Phytochemical characterization:

Chemical characterization was performed using an HPTLC chromatogram.

Mobile phase benzene: ethyl acetate (7:3) in formic acid 0.1 percent; scanning wavelength 366 nm. The STF-HAETR fraction that had not solidified was filtered, concentrated to 4 mL, and utilized for HPTLC.

In vitro **pro-inflammatory cytokine and nitric oxide evaluation:**

Histopaque-1077 (Sigma, Israel) gradient centrifugation was used to separate peripheral blood mononuclear cells (PBMCs) from the venous blood of healthy volunteers. The cells were washed and grown in RPMI-1640 media containing 10% foetal calf serum and 1% penicillin, streptomycin, and nystatin. In 1 mL of complete medium, 2×10^6 PBMCs were suspended. The cells were treated for 24 hours with 20 ng/mL lipopolysaccharide (LPS; Lipopolysaccharides W E. coli 055: B5) and CON (Concanavalin) A (2.5 µg/mL) to induce IL-12, TNF-a, and IL-6. STF-HAETR extract was added at graded concentrations (10, 25, 50, and 100 µg/mL) for 24 hours after LPS incubation. The cells were removed by centrifugation at 1500g for 10 minutes at the end of the incubation period, and the supernatants were collected. The cytokine content of IL-12, TNF-a, and IL-6 was determined using an ELISA technique and a standard calibration curve of relevant cytokines (BD pharmingen). Nitric oxide content was calculated using Griess reagent (Ding et al., 1988).

Assessment of pro-inflammatory mediators' iNOS (inducible nitric oxide synthase) by western blot analysis:

Mouse macrophage cell lines RAW 264.7 were used in the tests, which were grown in complete RPMI media with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated foetal calf serum. Cells were washed three times in PBS before extraction after stimulation with LPS (1 µg/mL) + IFN-γ (30 g/mL) and pre-treatment with STF-HAETR (34 µg/mL; IC_{50}) for 2 h. The whole-cell lysate (50 µg protein/lane) was electrophoresed on an 8% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel and electroblotted onto PVDF (polyvinylidene fluoride) membrane. Membranes were blocked with 5% BSA/TBST (Bovine Serum Albumin/Tris-buffered Saline and Tween 20) and probed in TBST with a polyclonal anti-mouse iNOS antibody (1:1000 dilution) and goat anti-mouse IgG conjugated to horseradish peroxidase (1:7000 dilution, Santa Cruz Biotechnology). ECL detection was performed as per manual instructions after heavy washing. As a loading control, the same membrane was stripped using stripping buffer and probed for bactin.

In-vitro **cyclooxygenase (COX) inhibition appraisal of STF-HAETR:**

The recombinant enzyme was used in a commercially available colorimetric COX (ovine) inhibitor screening test kit to measure COX-1 and COX-2 inhibitory activity. STF-HAETR was dissolved in 0.2 % DMSO and prepared shortly before use, with concentrations ranging from 10, 20, 50, and 100 µg/mL added to the reaction system. As a co-substrate with AA, N, N, N, N-tetraethyl-p-phenylenediamine (TMPD) was used to assess COX activity (reduction of PGG2 to PGH2). The oxidation of TMPD was measured spectrophotometrically at 590 nm using a 96-well plate reader. In control incubations with omitted enzymes or heat-denatured enzymes and without fraction in

conjunction with TMPD, no colorimetric change was seen. The linear regression analysis of the percent of COX inhibition vs. concentration curve was used to determine the IC_{50} value of STF-HAETR against COX-1 and COX-2 activities.

In vitro anti-asthmatic evaluation:

Adult goat trachea that had been freshly extracted was procured from a local slaughterhouse, and preparations for examination were made (Nag et al., 1974). The tracheal chain was suspended in a bath of Kreb's solution, which contained sodium chloride 6.9, potassium chloride 0.35, calcium chloride 0.28, magnesium sulphate 0.28, sodium bicarbonate 2.1, potassium dihydrogen phosphate 0.16, and glucose 2.0 gL-1, and was continuously aerated and maintained at 37^{0} C. According to the procedure, the entire preparation was used for the test (Dhonde et al., 2008). By maintaining a 5-minute time cycle, a dosage response curve for histamine was generated at various molar concentrations. After establishing a dose-response curve of histamine (2.5 µg/mL) in the organ bath of the trachea from 0.1 to 1.6 mL of Kreb's solution, 100 µg/mL of STF-HAETR was administered to each reservoir with a submaximal dosage of histamine. In the absence and presence of STF-HAETR, the dosage response curve of histamine was shown as a percentage of maximal contractile responses.

In vitro inhibition of protein denaturation

In vitro, a protein denaturation test was used to assess the anti-arthritic potential. The final reaction mixture (5 mL) was made up of 2.40 mL of a 5 percent aqueous bovine serum albumin solution and 0.10 mL of STF-HAETR (10, 25, 50, and 100 µg/ mL of final volume) dissolving in 0.2 percent DMSO (respect to final reaction volume). Instead of test fraction, 0.10 mL blank 0.2 percent DMSO was utilized in the untreated control group. A tiny quantity of 1 N HCl was used to alter the pH to 6.3. All of the samples

were heated at 57^0 C for 30 minutes after being incubated at 37 C for 20 minutes. To make up the final reaction volume, 2.5 mL phosphate buffer saline (pH 6.3) was added to each tube once the samples were cooled (5 mL). Turbidity was determined using a spectrophotometric method (Sadique et al., 1989) at 660 nm with positive controls containing aspirin (100 μg/mL) and untreated controls. According to the procedure used, the product control group lacked bovine serum albumin (Sadique et al., 1989).

The percentage inhibition of protein denaturation was calculated as follows:

Percentage of inhibition= 100- (OD of test -OD of product control) X 100/ OD of untreated control

Proteinase inhibitory action:

To determine the proteinase inhibitory potential of the test material (STF-HAETR), a trypsin-mediated casein degradation experiment was performed. This experiment was used to evaluate the *in vitro* the anti-inflammatory and anti-arthritic activity of STF HAETR *in vitro*. 0.06 mg trypsin was dissolved in 1.0 mL 25 mM tris–HCl buffer to create the assay (pH 7.4). STF-HAETR test sample solutions of 10, 25, 50, and 100 μg/mL (relative to final reaction volume (3 mL) were generated by dissolving in 0.4 percent V/V DMSO in 1 mL of aqueous solution and mixing with 1 ml of trypsin solution in 25 mM tris–HCl buffer (pH 7.4). The 2 mL reaction mixture was incubated at 37^0 C for 5 minutes before adding 1.0 mL of 0.8 percent (w/v) casein. At 37 C, the complete reaction mixture was incubated for another 20 minutes. Instead of the test fraction, a 1 mL blank solution of 0.4 percent V/V DMSO was utilised as an untreated control group. To stop the enzymatic process, 2.0 mL of 70% (v/v) perchloric acid was added to each reaction mixture tube. A centrifuge was used to separate the hazy suspension. The absorbance of each group of the solution's supernatant was measured at

280 nm against a blank buffer (Oyedapo et al., 1995). The % proteinase inhibition was computed using the formula below and compared to positive aspirin (100 µg/mL) controls.

% of inhibition = 100 – [(OD of test solution - OD of only STF- HAETR fraction without casein - trypsin enzyme reaction) / (OD of untreated control) X 100].

 Membrane stabilization studies:

To imitate the *in vitro* anti-arthritic evaluation, an RBC membrane stability experiment was performed. The approach was used to stabilize the membrane of a human red blood cell (RBC) by inducing membrane lysis using a hypotonic solution. 2 mL hypotonic saline (0.25% NaCl), 1 mL 0.15 M phosphate buffer saline (pH 7.4), and 1.0 mL test sample solution of STF-HAETR [10, 25, 50, and 100 µg/ mL of final volume (4.5 mL)] dissolving in 0.2 % DMSO v/v solution in aqueous base A 0.5 mL HRBC suspension in normal saline (10% v/v) was added. Instead of test fraction solution, 1 mL of 0.2 percent DMSO v/v solution in an aqueous base was utilised for the untreated control group, whereas the product control group did not have red blood cells suspension. The finished reaction mixture volume was incubated for 30 minutes at 56^0C. For 20 minutes, all of the tubes were cooled under running tap water centrifuged the mixtures, and measured the absorbance of the supernatants at 560 nm (Brown et al., 1968).

Percent membrane stabilizing activity was calculated as follows:

Percent stabilization = 100 - (OD of test fraction - OD of product control)/OD of untreated control X 100

The untreated control represents 100% lysis by inducing 2 ml of the hypotonic solution of 0.25% NaCl. The result was compared with the positive control of aspirin (100 µg/mL).

Experimental animals:

In this study, albino mice of either sex (20-25 gm weight) and male albino rats (150-230 gm) were used. For all animal experiments prior approval of animal ethical committee was taken and experimental procedures were conducted as per the guidelines

***In vivo* assessment of anti-nociceptive pain of STF-HAETR:**

50 mice (20–25 g) were given 0.6 percent glacial acetic acid intraperitoneally by 10 mL/kg dosage basis twenty-four hours before the actual experiment. To assess the peripheral anti-nociceptive pain impact, the animals were observed for writhing movements (Collier et al., 1968). To cause abdominal constriction, 0.6 percent acetic acid was utilized. The next day's test was limited to individuals who showed one or more types of writhing motions (positive responders). The positive responders were given STF-HAETR at doses of 30 and 60 mg/kg non-lethal oral dosage on the test day. The test drug was diluted in a 0.2(M) phosphate-buffered saline solution containing 0.2 % tween 80 and 0.5% sodium CMC (carboxymethyl cellulose) and given intraperitoneally as a single dose half an hour before the glacial acetic acid challenge. Indomethacin, at a dosage of 5 mg/kg, was utilised as a positive control and was given orally in a single dose. In 0.2 (M) phosphate-buffered saline containing 0.2 percent tween 80 and 0.5 percent sodium CMC, indomethacin was dissolved. Following the injection of glacial acetic acid, each mouse was watched for 15 minutes for the total number of stretching episodes or writhing. The average number of writhes due to abdominal constriction for each mouse in each of the six experimental groups was recorded. In comparison to untreated controls, the percentage of protection for two distinct dosages was assessed (receiving glacial acetic acid only without additional drugs). Each treatment group's number of writhes was compared to the number of

writhes in the untreated control group. The number of writhes was counted, and the protection % was computed using the method below.

% protection = (Mean writhes in control untreated group - mean writhes in treated group/ mean writhes in control) X 100

Anti-nociceptive pain assessment by the hot-plate technique:

Swiss albino mice (25–35 g) of either sex was used in the experiment, which was randomly selected on a hot-plate apparatus (53 ± 0.5^0C). The reaction time was measured from the moment the animal was placed on the hot plate to the time it licked its hind paw or leaped off. For comparison and validation of the model, 20 mg/kg aceclofenac was delivered intraperitoneally for one time as a positive control, standard analgesic medication. STF-HAETR was administered orally as a single dosage at 30 and 60 mg/kg for one time. Following placing the tested animal on Eddy's hot plate, the reaction time was measured in seconds; how long does it take for the animal to lick its paw for the first time after treatment. By measuring the number of paw licks in each experimental group, the central analgesic effect was represented as a percentage of protection in the test group compared to untreated control groups (Turner 1965). The untreated control group was administered a liquid solution of 0.2(M) phosphate-buffered saline containing 0.2% tween 80 and 0.5% sodium CMC without STF-HAETR as the only vehicle.

In vivo **anti-inflammatory evaluation:**

With minor modifications to the carrageenan-induced rat hind paw edoema model, the procedure was used as reported (Winter et al., 1962). Before the trial, albino rats weighing between 150 and 180 g have fasted for 18 hours. The creatures were weighed, identified, and separated into four groups, each of which had six animals. Edema was generated in all rats' left hind paws by injecting 0.1 mL of 1 percent (w/v) carrageenan

in distilled water subcutaneously into their footpads. The first group was retained as a control group and given the vehicle's volume (0.2 M PBS). STF-HAETR was given to the second and third groups at doses of 50 and 100 mg/kg, respectively. 1 hour before the injection of carrageenan. The last group (standard) got indomethacin intraperitoneally at a dosage of 10 mg/kg (Mino et al., 2004). Each rat's paw volume was measured using a mercury plethysmometer immediately before carrageenan injection, as well as 1, 2, and 3 hours later. Each group's edoema and inhibition rates were calculated as follows:

Edema rate (E) % = Vt - V0/V0 X 100

Inhibition rate (I) % = Ec Et/Ec X 100

where

V0 is the volume before carrageenan injection (mL),

Vt is the volume at t hours after carrageenan injection (mL),

Ec, Et is the edema rate of the control group and treated group, respectively.

Acute toxicity study and liver, kidney, and heart marker enzyme analysis:

30 rats were divided into five groups of six rats each at random. STF-HAETR dosages ranging from 100 to 2000 mg/kg were given to four groups. Intraperitoneally, Group 1 received a liquid solution of 0.2(M) phosphate-buffered saline with 0.2 percent tween 80 and 0.5% sodium CMC (carboxymethyl cellulose). Total STF-HAETR (100, 500, 1000, and 2000 mg/kg, respectively) was administered intraperitoneally to groups 2–5. Similarly, the toxicity investigation was carried out with 30 mice, who were divided into five groups, each with six animals. The test mice were given intraperitoneal doses of 60, 120, 400, and 800 mg/kg in four groups. Group 1 was given a 0.2 (M) phosphate-buffered saline solution containing 0.2% tween 80 and 0.5% sodium CMC

(carboxymethyl cellulose). Over two months, all of the animals were monitored for a death rate. After 2 months of surveillance, serum enzymes such as urea, creatinine, SGPT, SGOT, ALT, and blood LDH were measured.

Statistical analysis:

GraphPad Prism 5.0 version software was used for statistical analysis. IC50 was estimated using nonlinear regression analysis. In other situations, the differences between the treatment groups and the untreated controls were estimated using an unpaired student t-test. P values less than 0.05 were deemed statistically significant. All of the data was presented as a mean with standard deviation (SEM).

I.   Evaluation of Gelsemium *sempervirens* against Dementia

This investigation determines the potential of reconstituted *Gelsemium* sempervirens mother tincture on oxidative stress processes, memory as well as learning dysfunctioning in a scopolamine-induced dementia mouse model, all of which are thought to be symptoms of cholinergic and secretase dysfunction or senile CNS dysfunction in dementia (Drever et al., 2007).

Chemical profile of major phyto-components present in the *Gelsemium* mother tincture:

Upon examination, the *Gelsemium* mother tincture contained Alkaloids and Coumarin. the Alkaloids found were Gelsemine (8% w/w), sempervirine (1.6% w/w) while Scopoletin (1.67% w/w), and Scopolin (0.8% w/w) type of coumarins were found. It also contains 1.31 % w/w Gelsemine as Iridoid (Søren et al.,1987).
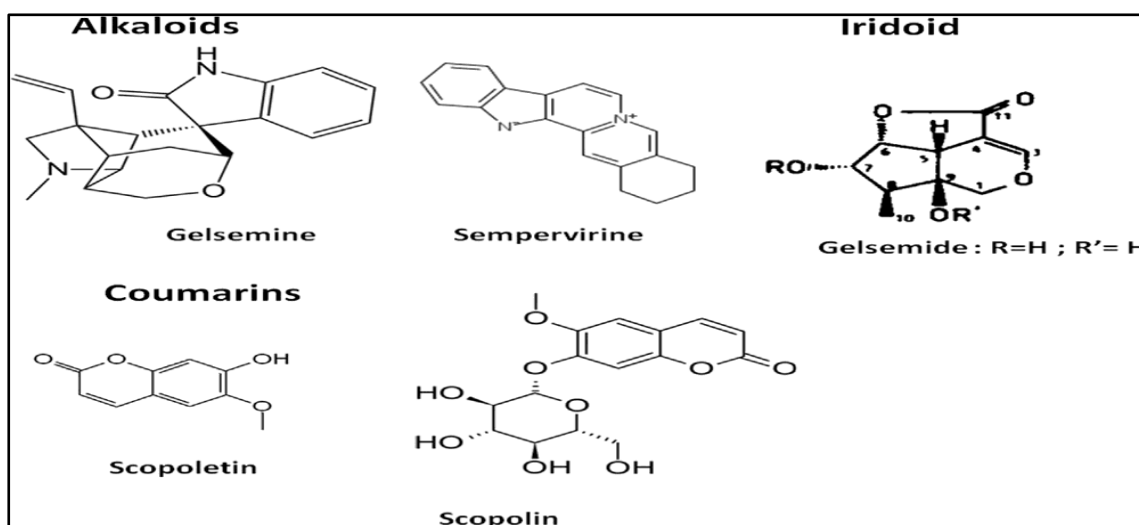
Fig. 5.1: Phytochemical component present in *Gelsemium sempervirens*.

Effect of reconstituted *Gelsemium* tincture on ex vivo acetylcholinesterase (AChE) activity

In a dose-dependent *in vitro* acetylcholine esterase inhibition study illustrated that

reconstituted *Gelsemium sempervirens* tincture significantly reduced AChE produced from brain tissue homogenate in contrast to untreated controls, as shown in **Fig.5.2a**. When compared to untreated controls, it displayed % AChE inhibition at 75 mg/ml having IC_{50} of 9.5 µg/ml (P<0.001), whereas galanthamine, a conventional inhibitor, had an IC_{50} of 4.73 µg/ml on *in-vitro* AChE inhibition (*P* <0.001).
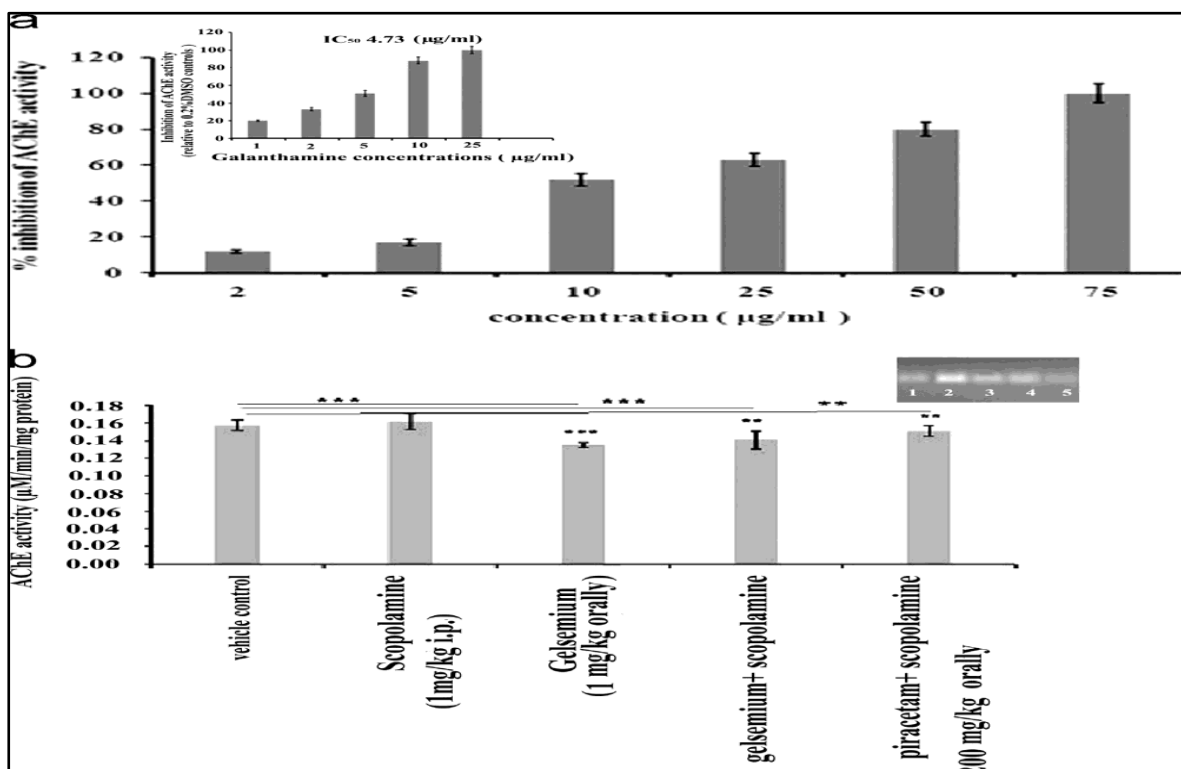
Fig.5.2: The efficacy of reconstituted mother tincture of Gelsemium sempervirens on AChE inhibitory effect in mice brain at concentrations dependent (5.2a). and in the brain of scopolamine-treated amnesic mice (5.2 b).

In vivo **AChE inhibition study at post-treatment of Gelsemium:**

In comparison to scopolamine-treated dementia mice, short-term (1 day) therapy with Gelsemium (1 mg/kg p. o.) led in substantial decrease in particular AChE potential [F (1,10) =12, P<0.01]. *In vivo* AChE inhibition was better in the Gelsemium-treated mice group than in the typical nootropic medication piracetam-treated animals' group [F

(1,10) =12, P <0.01]. *Gelsemium* outperformed typical memory enhancer medications like piracetam, demonstrating in vivo AChE inhibition even at 200 times lower doses. These findings were validated by examining mRNA expression levels in mouse brains, which revealed that AChE mRNA expression was considerably elevated in just the scopolamine-injected animals' group **(Fig.5.2b)**. In contrast to solely scopolamine-treated dementia mice, Gelsemium pre-treatment significantly reduced AChE mRNA expression in scopolamine-injected mice (Fig. 2b inset; lane 3). In comparison to the positive controls (piracetam) treated mice brain homogenate, the Gelsemium treated group showed considerable suppression of AChE mRNA expression **(Fig. 5.2b)**.

True and false-positive activity assessment by Gelsemium through TLC bioautographic assay for AChE inhibition study

TLC bioautographic assay was used to examine the inhibitory activity of AChE in the reconstituted Gelsemium tincture that exhibited the most promising activity by enzyme assay spectrophotometrically. This established whether the response is due to real inhibition by the enzymes or not. All of the polar compounds separated by TLC chromatogram were confirmed to be real AChE inhibitors **(Fig.5.3a)** and similar to galanthamine, a typical positive control inhibitor of AChE. **(Fig. 5.3a).** White spots of inhibition for the strong active Gelsemium tincture extract **(Fig. 5.3a)** and galanthamine were monitored instead of false-positive results **(Fig. 5.3b).**
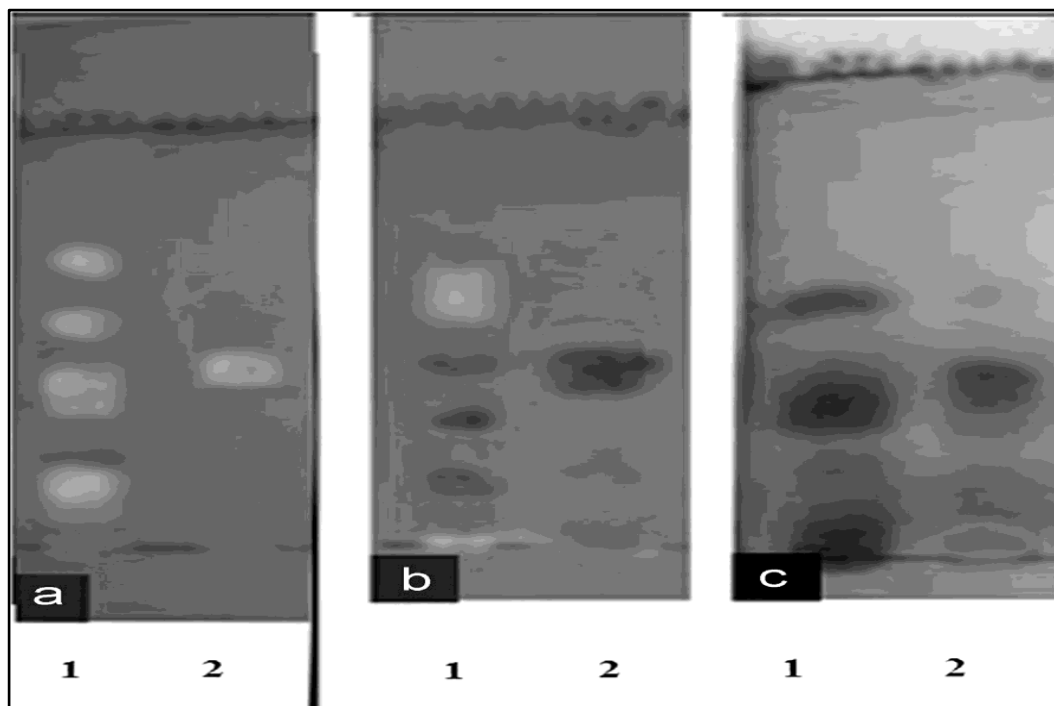
Fig. 5.3. The presentation of true and false positive inhibition of acetylcholine

esterase by reconstituted Gelsemium tincture extract.

In above **Fig. 5.3** The silica gel TLC layer was loaded with lane 1: Gelsemium extract (20 mg/ml) lane 2: galanthamine (10 mg/ml). The mobile phase was taken as chloroform: methanol (9:1). (a) White spots in the yellow background correspond to true enzyme inhibition while 5 mM ATCI and DTNB (dissolved in 50 mM of Tris HCl buffer, pH 8 and 37 1C) are sprayed onto the TLC chromatogram layer and followed by 3 U/mL of AChE (50 mM of Tris HCl buffer, pH 8 and 37^0C). (b) White spots in the yellow background represent the false-positive inhibition when the layer was sprayed with 5 mM ATCI and DTNB, dissolved in 50 mM of Tris HCl buffer, pH 8 and 37^0C to the TLC chromatogram layer. (c) The similar chromatogram was sprayed with 2% antimony trichloride in 10% methanolic KOH reagent to display the true AChE inhibiting polar compounds.

In vitro BACE1 inhibition

The inhibitory action of AChE in the reconstituted *Gelsemium* tincture that displayed the most promising activity by enzyme assay spectrophotometrically was investigated using a TLC bioautographic test. This determined whether true enzyme inhibition is responsible for the reaction or not. All of the polar compounds separated by TLC chromatography were proven to be true AChE inhibitors and comparable to galanthamine, a common AChE positive control inhibitor. (Fig. 5.3a.) Instead of false-positive results, white spots of inhibition for the highly potent Gelsemium tincture extract (Fig. 5.3a) and galanthamine were observed (Fig. 5.3b). Gelsemium improved acetylcholine neurotransmission in the hippocampus by inhibiting the AChE enzyme, decreasing BACE1 activity, and up-regulating the GSH content to protect dementia mice against cognitive and memory impairment.

Estimation of reduced glutathione level in mouse brain

To investigate the impact of *Gelsemium sempervirens* tincture on brain oxidative status, reduced glutathione levels at post-treatment of Gelsemium in scopolamine-induced dementia mice were assessed. As observed in Fig.3b, the levels of reduced glutathione significantly increased (31%) in the brain tissue homogenate after Gelsemium treatment (1 mg/kg, p.o. in which the jasmine was present as 0.08 mg/kg (data not shown) for 14 days) in comparison to control mice [$F_{(1,10)}$ =73.973, $P<0.001$]. Interestingly, GSH content is higher in Gelsemium treated mice compared to piracetam treated scopolamine-induced dementia mice [$F_{(1,10)}$ =4.138, $P<0.069$]. Gelsemium treatment significantly [$F_{(1,10)}$ =13.407, $P<0.004$] enhanced the GSH level as compared to vehicle controls. Previous reports suggests that hippocampus AChE has a function in cognitive performance modulation. It's an important part of dementia-related memory

loss and the fundamental cause of cognitive problems (Das et al.,2005). As a result, inhibiting AChE is a promising treatment option for dementia and related cognitive problems (Terry et al., 2003). Our study also validated the earlier concepts regarding the management of dementia demonstrating in vitro reduction as well as promising in vivo inhibition of AChE activity by Gelsemium.

Effect of reconstituted Gelsemium tincture on behavioral models

Elevated plus-maze study

The investigation was performed to determine the potential role of the Gelsemium on scopolamine-induced memory impairment, we conducted behavioral studies measuring the TL (second) of mice in an elevated plus-maze model. Results demonstrated that after 14 days of treatment with the reconstituted Gelsemium sempervirens mother tincture at 1 mg/kg, the tested mice regained their loss of memory function of almost 60% [F (1,10) =100.38, P<0.001] against scopolamine-induced dementia, while standard nootropic drug piracetam (200 mg/kg i.p.) showed 70% [F (1,10) =132.165, P<0.001] learning improvement over untreated scopolamine-induced amnesic group (Fig. 5.4).
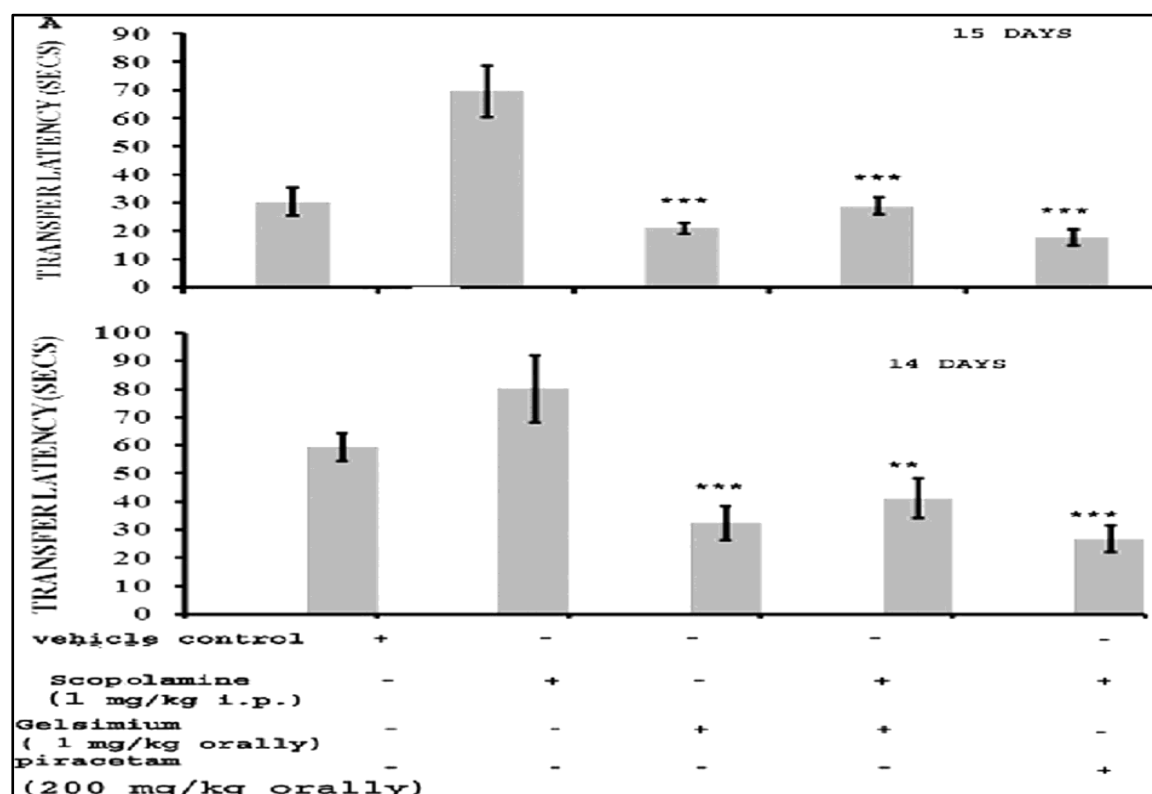
Fig. 5.4: Presentation of the anti-dementia effect of reconstituted mother tincture

of *Gelsemium sempervirens*.

The 14th day of Gelsemium therapy demonstrated development in the animals' learning behaviour, whereas the next day's transfer latency reflected information or memory retention, resulting in a shorter transfer latency time than the 14-day observation. Both Gelsemium (1 mg/kg, oral) and piracetam (200 mg/kg, i.p.) significantly [F (2,15) =108.956, P<0.001] reduced the transfer latency time in comparison to scopolamine treated memory-impaired mice.

Passive avoidance study

This test with step-down delay was utilised to assess the improvement of dementia-mediated memory loss and learning difficulties following Gelsemium treatment. The scopolamine-treated mice had a substantially lower step-down latency time (s) than the vehicle control animals [F (1,10) =138.922, P <0.001]. When scopolamine-treated mice

were given Gelsemium (1 mg/kg p.o.) for 14 days, step-down latency was significantly

increased on day 15 (**Table 5.1**) compared to untreated controls [F (1,10) =145.494,

P<0.001]. Gelsemium's learning-improving activity was virtually identical to that of the

typical nootropic medication piracetam. Furthermore, in the passive avoidance test, the

step-down latency time of just Gelsemium-treated mice was better than the vehicle-

treated mice group. Gelsemium's memory-enhancing properties were discovered.

**Table 5.1: Effects of reconstituted *Gelsemium sempervirens* mother tincture on
scopolamine-induced PA retention deficits in mice, as measured by step-down
latency**

Step down latency (s)	Vehicle controls	Scopolamine (1 mg/kg i.p.)	Gelsemium (1 mg/kg orally)	Gelsemium + Scopolamine	Piracetam (200 mg/kg i.p.) +Scopolamine
	41.16 ± 3.5	17 ±3.6	58±85	43.5 ±4	71.5 ±9

Previous research has revealed that hippocampus AChE has a function in cognitive

performance modulation. It's an important part of dementia-related memory loss and

the fundamental cause of cognitive problems (Das et al., 2005). Gelsemium induced

suppression of AChE activity via *in-vitro* and *in-vivo* was also demonstrated in our

investigation, confirming prior thoughts about dementia care. Our findings imply that

there is a link between AChE inhibition *in-vitro* and *in-vivo*. It may also improve

cholinergic activity by increasing the amount of acetylcholine in cholinergic synapses

in the brain (Lane et al., 2006), hence alleviating cognitive and memory dysfunctions in

dementia-related neurodegenerative illness. Our results show that Gelsemium-induced

AChE inhibition is attributable to polar molecules in the chromatogram, not a false

positive impact in TLC-based suppression of the chemical interaction between

thiocholine and DTNB. The real enzyme inhibition appears to be elicited by the alkaloid, iridoid, and coumarin-like substances found in the polar area of the chromatogram. However, we evaluated the mRNA expression level of AChE inhibition using Gelsemium, which does not suffer from the problem of false-positive effects caused by carbonyl, aldehyde, or amine function containing chemicals that interfere with Ellman's reagent's color response. As a result, the polar fraction would be chosen for further separation of particular AChE inhibitors as revealed by this study.

The findings of the raised plus maze and passive avoidance tests in mice produced by Gelsemium show that amnesia is improved, learning skills are improved, and cognitive impairment is reduced. In both exteroceptive behavioral models, 1 mg/kg of reconstituted Gelsemium tincture fed for 14 days significantly restored scopolamine-induced impairment of spatial memory tasks, whereas standard memory enhancers such as *Bacopa monnieri* and *Ginko biloba* plant extract at 30 and 60 mg/kg, respectively, showed similar cognitive enhancing activity (Das et al., 2002). According to the behavioural investigations, Gelsemium-treated dementia mice had an exceptional memory and learning function retention.

These findings suggest that a low dose of Gelsemium sempervirens reconstituted mother tincture may offer an emerging therapeutic option for the prevention of dementia and related neurodegenerative disorders by improving learning, memory deficits, and brain oxidative damage in scopolamine-induced amnesic mice. Dual inhibition of AChE and BACE1, as well as activation of the endogenous antioxidant (GSH) defense system, corroborate these findings. At the experimental therapeutic dosage, gelsemium had no evident adverse effects or mutagenicity in mice. As a result, Gelsemium might be a potential pharmacological option for dementia and

related neurodegenerative illnesses. However, more research is needed to determine the

precise polar active ingredients or bioactive fractions of the Gelsemium tincture that are

responsible for its anti-dementia efficacy, as well as the underlying mechanism.

II. Evaluation of *Trigonella foenum-graecum*, *Piper betel*, and *Lagenaria siceraria* homogenate against arthritis and asthma

Extractive yield, Polyphenol content, antioxidant activity, and phytochemical screening study

Table 5.6 shows the results of a qualitative phytochemical screening test, polyphenol concentration, antioxidant activity, and % yield. The studied aqueous extracts of fenugreek and bottle-gourd included therapeutically active phyto-metabolites such as saponin, glycosides, tannin, flavonoid, mucilage, protein, aromatic and aliphatic amino-acids, as shown in the table. Betel leaf extract, on the other hand, contains tannin, flavonoids, protein, mucilage, aromatic, and aliphatic amino acids. Betel leaf and fenugreek samples both contain lignin. In comparison to initial dry crude materials, fenugreek, bottle gourd, and betel leaf extract yielded 2, 2.12, and 5.5 % w/w extractive yield, respectively. The amount of polyphenols is an essential metric for determining anti-oxidant action (Awe et al., 2013; Nakajima et al., 2017). It is also important in the denaturation of proteins (Caillet et al., 2011). The highest polyphenol content was found in betel leaf, followed by fenugreek and bottle gourd, according to the data. Furthermore, the antioxidant activity of betel leaf, fenugreek, and bottle gourd, respectively, is 71%, 33%, and 12%.

Table 5.2: Phytochemical test and percentage yield of fenugreek, bottle gourd, and betel leaf in aqueous fraction and homogenate

Test	Piper betel	Fenugreek	Bottle gourd	Aspirin	Diclofenac Sodium	Azelastine
Polyphenol	+ Ve	+ Ve	+ Ve	NTD	NTD	NTD
Saponin	-Ve	+Ve	+Ve	NTD	NTD	NTD
Xanthroproteic test	+Ve	+Ve	+Ve	NTD	NTD	NTD
Ninhydrin test	+Ve	+Ve	+Ve	NTD	NTD	NTD
Flavonoid	+Ve	+Ve	+Ve	NTD	NTD	NTD
Glycoside	-Ve	+Ve	+Ve	NTD	NTD	NTD
Lignin	+Ve	+Ve	-Ve	NTD	NTD	NTD
Mucilage	+Ve	+Ve	+Ve	NTD	NTD	NTD
Percentage yield of water extract (%)	5.5	2	2.12	NTD	NTD	NTD
Polyphenol content (mg GAE/100 ml)	115	54	17	NTD	NTD	NTD
Antioxidant activity (%)	71	33	12	NTD	NTD	NTD
IC_{50} for protein denaturation (µg/ml)	95	92	200	-	125	-
IC_{50} for proteinase inhibition (µg/ml)	107	100	131	99	-	-
IC_{50} for RBC membrane stabilization (µg/ml)	100	113	96	-	-	-
IC_{50} for 15-Lox (µg/ml)	38	40		-	-	35

NTD: Not tested

HPLC characterization of bioactive fractions of raw phytoextract

Fenugreek has some main peaks, which correlate to the concentrations of gallic acid and isoflavones, as seen in Fig. 5.5 (a). The HPLC characterization of betel leaf is shown in Fig. 5.5 (b). The peaks show the presence of chlorogenic acid and hydroxychavicol, two of the betel leaf's most important bioactive parts. The bottle

gourd bought from IIT Kharagpur market is of the sweet kind and does not contain

cucurbitacin B, as shown in Figs. 5.5 (c) and (d).
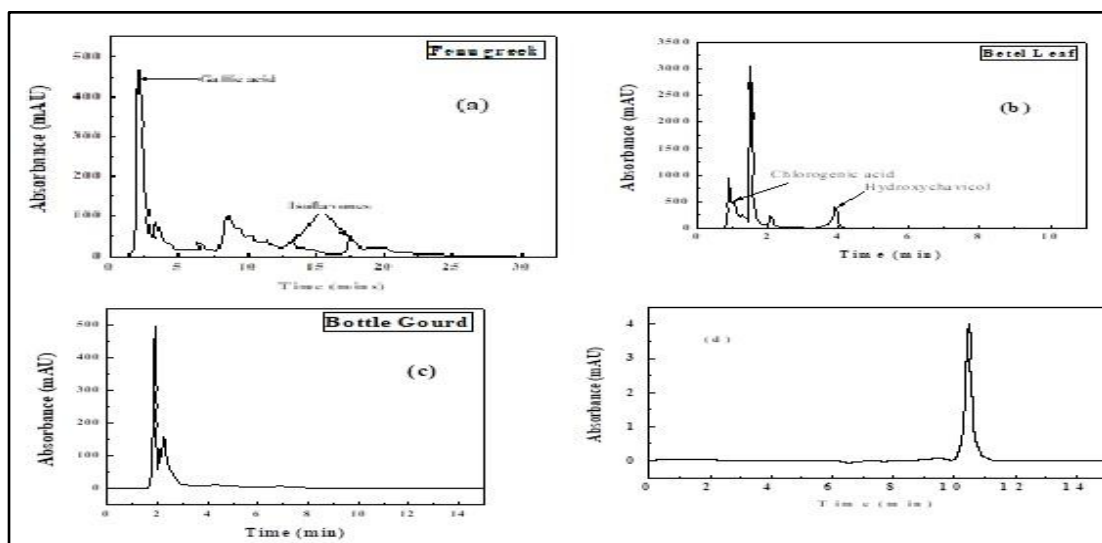
Fig. 5.5: HPLC profile of (a) fenugreek, (b) betel leaf, (c) bottle gourd, and (d)

pure cucurbitacin B.

In vitro **BSA denaturation inhibition and proteinase inhibition study**

Table 5.3 and Fig 5.6 show the inhibitory effect of fenugreek, betel leaf, and bottle-

gourd extracts on BSA protein denaturation as well as their anti-proteinase activity test.

At graded dosages, fenugreek and betel leaf showed excellent protection against a heat-

induced protein denaturation cascade. In terms of protein denaturation inhibitory action,

fenugreek and betel leaf had substantial IC50 values of 90 and 95 µg/ml (Table 5.2).

The inhibitory effects of Fenugreek and Betel leaf on protein denaturation were 1.34

and 1.26 times more efficient than the conventional positive inhibitor, diclofenac

sodium (IC50: 120 µg/ml; Table 5.3).

Table 5.3: IC$_{50}$ values of test extract and reference standard drug (azelastine) against 15-LOX inhibition assay.

Test sample	IC$_{50}$ against 15-LOX inhibition assay
Aqueous fraction of betel leaf	20
Bottle gourd homogenate	23
Azelastine	15

At 200 µg/ml, fenugreek showed 91% inhibition of BSA protein denaturation, whereas the typical inhibitor diclofenac sodium showed only 87% anti-protein denaturation action. In comparison to fenugreek, betel leaf extract inhibited protein denaturation by 87 %, 51%, and 28% at 200, 100, and 50 µg /ml, respectively. In comparison to the usual positive inhibitor, diclofenac sodium, lesser concentrations (50 and 100 µg /ml) of fenugreek and betel leaves showed excellent protective efficacy. Fenugreek and betel leaves had approximately the same impact as the conventional inhibitor at 200 µg /ml. The presence of polyphenols such as phytochemicals in fenugreek and betel leaf may explain their heightened anti-protein denaturation action, which is ascribed to their antioxidant content and anti-arthritic activity (Lee and Kim., 2010). This is substantiated by the fact that there is a strong link between protein denaturation inhibition and medicinal drug anti-oxidant properties (Badii and Howell., 2002). Previously, it was thought that protein denaturation caused by the auto-antigen synthesis in certain rheumatic disorders was main cause of RA (Williams et al., 2008). Joint cartilage protein is denatured in arthritic inflammatory conditions as a result of pro-inflammatory cytokines (TNF and IL-6) production at higher level -6). As a result, fenugreek and betel leaf extracts might be prospective anti-arthritic agents, as they exhibit promising anti-denaturation effects, which could be due to the high tannins and polyphenolic components such as flavonoids, as shown in **Table 5.2**.

In continuation of *in vitro* anti-arthritic assessment of the experimental extracts, the anti-proteinase activity assay of fenugreek, betel leaf, and the bottle-gourd extract was presented in **Table 5.3**.

Fenugreek and betel leaf demonstrated significant inhibitory action on α-chymotrypsin proteinase enzyme activity with an IC_{50} of 95 and 98 µg/ml (Table 5.3), respectively, in a dose-dependent manner. The maximum inhibition (92%) against α-chymotrypsin proteinase mediated β-casein degradation was detected by fenugreek at 200 µg/ml. However, bottle gourd showed moderate to strong inhibition against this serine protease family member, an α-chymotrypsin enzyme with an IC_{50} of 115 µg/ml (**Table 5.3).** Over-expression of proteinases displays very important activity in damage of cartilage as well as tissue joints in arthritis. Fenugreek and betel leaf could be a promising anti-proteinase agent for eliciting the anti-arthritic role. Therefore, it can be assumed that fenugreek and betel leaf are effective against the inflammatory syndrome by suppressing the TNF and IL-6 at synovial levels. Our *in vitro* results executed by Betel leaf and fenugreek aqueous fractions are quite relevant to the proposed activity. Moreover, polyphenols may enhance the antioxidant activity of betel leaf and fenugreek via flavonoids-protein interaction to display the potential anti-inflammatory as well as anti-arthritic potential via proteinase inhibition as per previously published reports (Rauf et al. 2015, Palit et al., 2018; Palit et al., 2016). Polyphenols were found to be reported as serine protease inhibitors which may act as potential anti-inflammatory as well as anti-arthritic agents (Viskupicova et al., 2012).
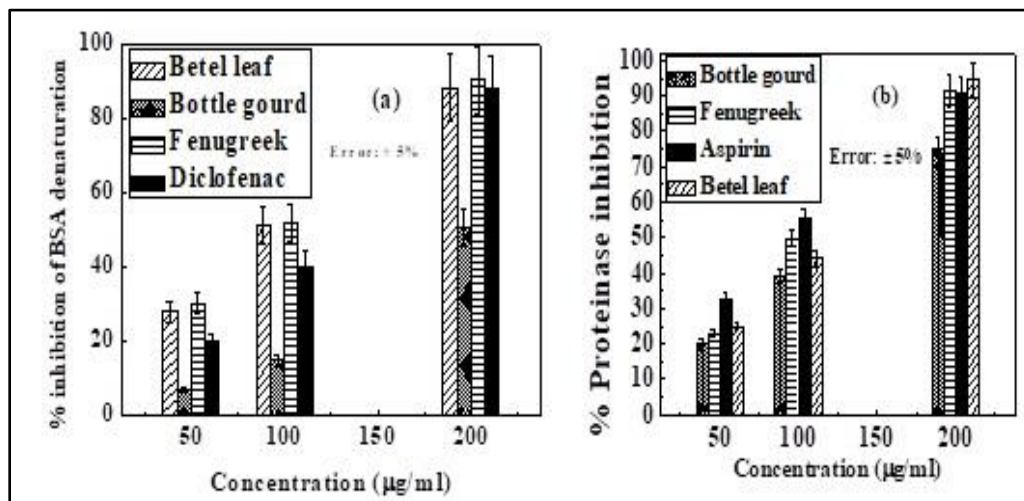
Fig. 5.6: (a) BSA denaturation inhibition assay; (b) Anti proteinase activity assay

in trypsin mediated β-casein degradation model.

Membrane stabilization study, 15-lipoxygenase (LOX) inhibition, and *in vitro* anti-asthmatic bioassay study

The RBC membrane stabilization test for bottle gourd, fenugreek, and betel leaf is shown in Table 3. Betel leaf and fenugreek extract stabilized hypotonic saline caused human RBC membrane lysis by 97% and 83% at 200 µg/ml, respectively, with IC50 of 105 and 128 µg/ml (Table 3), whereas bottle gourd provides 75% protection at an equal dose (Table 3). Our findings show that a lower concentration of bottle-gourd (100 µg/ml) produced better effects than a greater quantity (200 µg/ml). As a result, the IC50 of bottle-gourd has been determined to be 100 µg/ml (Table 3).

The approach of stabilizing RBC membranes is useful for determining the mechanisms of the anti-inflammatory effects of various phytochemicals. Heat-induced hemolysis was well-protected by all three fractions. 15-LOX inhibition research was carried out with betel leaf and bottle gourd extract to determine anti-inflammatory and anti-asthmatic action, findings are shown in Fig. 5.7. Study on histamine-induced

tracheal bronchoconstriction and its protective effect using bottle gourd extract was carried out.

With IC50 values of 20 and 23 µg/ml, respectively, betel leaf extract and bottle gourd homogenate inhibited 15-LOX enzyme activity in a dose-dependent manner (Table 3). The inhibitory effects of the bottle-gourd homogenate and betel leaf extract (Table 2) are almost identical to those of the conventional 15-LOX inhibitor azelastine (IC50 15 µg /ml). At 100 µg /ml, bottle gourd and betel leaf extracts inhibited 15-LOX by 98 and 96 % respectively, whereas Azalastine, a conventional medication, inhibited 15-LOX by 99.5%.
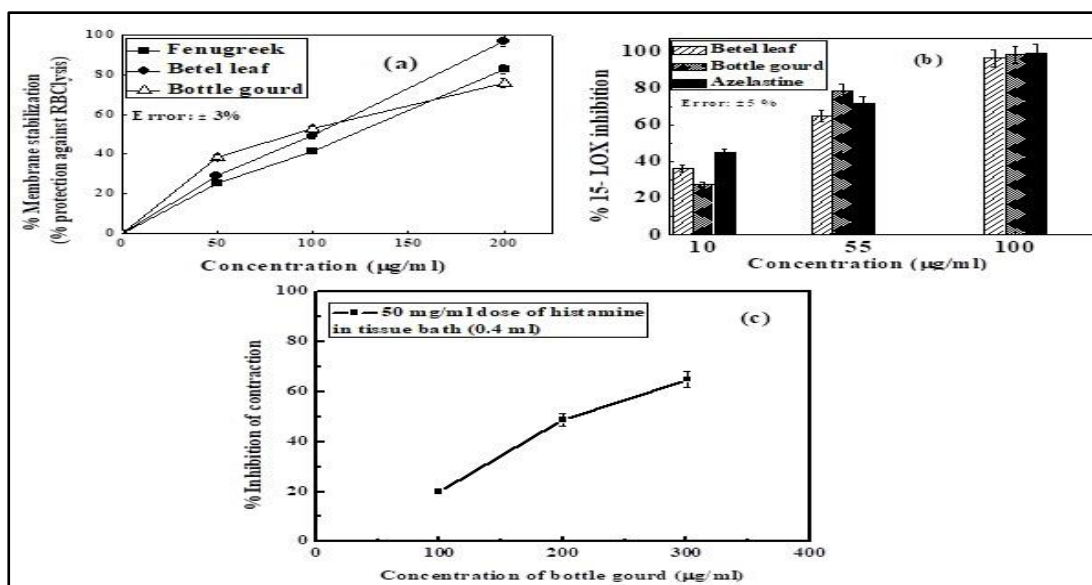
Fig. 5.7: (a) RBC membrane stabilization assay; (b) 15-Lipoxygenase inhibition assay; (c) percentage inhibitory contractile response in goat tracheal bioassay in response to histamine-induced contraction.

Since bottle gourd and betel leaf are excellent inhibitors of 15-LOX, these two agents could be considered for the management of asthma and other related inflammatory complications, as they reduce the formation of cysteinyl LTs (cysLTs), a potent bio-active inflammatory mediator and bronchoconstrictor produced by the lipoxygenase

pathway of arachidonic acid metabolism. As a result, betel leaf and bottle-gourd extract might be synthesized as lipoxygenase inhibitors for a new asthma therapy regimen ranging from acute inflammatory to chronic progressive (Abe and Gunji., 2004).

The bottle-gourd extract was used in a bio-assay in a histamine-induced bronchoconstriction model in the goat tracheal chain to determine its anti-asthmatic activity. An in-vitro bio-assay revealed that bottle-gourd extract reduced histamine-induced bronchoconstriction. This study found that bottle-gourd extract reduced contractions elicited by histamine at 50 µg/ml in 0.4 ml tissue bath by 20%, 48.7%, and 65 percent in a dose-dependent manner at 100, 200, and 300 µg/ml concentrations, respectively.

In vitro pro-inflammatory cytokine inhibitory assessment:

The levels of IL-6 and TNF- were considerably raised in supernatants of human peripheral blood mononuclear cell (PBMC) derived macrophages treated with Lipo-poly saccharide in the culture (Fig. 5.8). Fenugreek and betel leaf extract were tested for their ability to reduce pro-inflammatory cytokine production. As a control, human PBMC were grown in a 0.02 M phosphate buffer solution. Lipopolysaccharide (LPS) was used as a positive regulator to boost TNF-α and IL-6 levels, imitating arthritis-like inflammation (Ganju et al., 2003). At 200 µg/ml, extracts from fenugreek and betel leaf lowered the levels TNF-α and IL-6 as seen in Figs. 8(a) and (b). TNF-α, the most powerful pro-inflammatory cytokine, was suppressed by fenugreek and betel leaf extract, respectively, at 200 µg /ml, by 49% (P< 0.0001) and 37% (P< 0.001) (Fig. 5.8b). In comparison to untreated controls, they also reduced the production of another pro-inflammatory cytokine, IL-6, from cultured PBMC cells (Fig. 5.8a). The results showed that 200 µg /ml dosages of fenugreek and betel leaf extract reduce IL-6

production in LPS stimulated PBMC cultured cells by 36% (P< 0.001) and 30% (P <0.001), respectively (Fig. 5.8a).
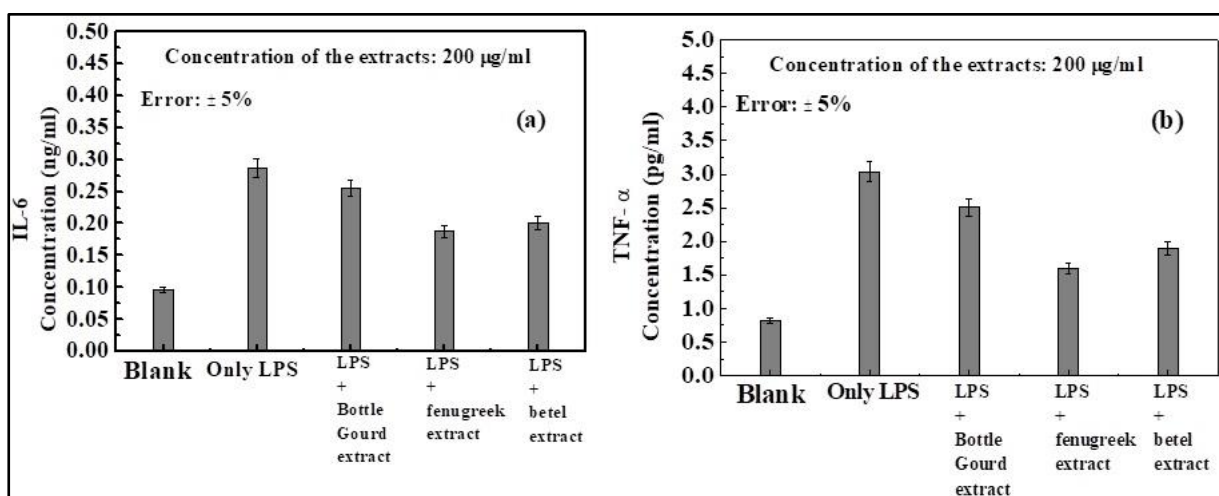
Fig. 5.8: Pro-inflammatory cytokinin inhibition for (a) interleukin-6 and(b)TNF- α

The considerable decrease of TNF-α and IL-6 by the test extract might be a possible role in the suppression of leukocyte migration and protection against disorders such as asthma and arthritis. In vitro, TNF-α may up-regulate the production of pro-inflammatory cytokines like IL-1 and IL-6, which play a key role in RA patients' joint inflammation, cartilage, and bone degeneration (Carteron., 2000). It also induces synoviocyte hyperplasia and the production of extracellular matrix-degrading enzymes and chemokines, hastening the arthritic erosion process (McInnes and Schett., 2007).

As a result, during severe acute bronchial asthma, elevated levels of TNF-α and IL-6 are also focused on destroying bronchial tissues (Ammon., 2016). IL-6, one of the most significant pathophysiological agents in bone resorption, promotes the production of autoantibodies such as rheumatoid factor (RF) in an unspecific manner. As a result, it is fair to presume that fenugreek and betel leaf extract might be used as immunosuppressive drugs for treating arthritis. Due to their TNF-α and IL-6

suppressing properties, bottle gourd homogenate, and betel leaf extract were shown to be helpful against asthmatic diseases, as shown in **Fig.5.7.**

Fenugreek and bottle gourd are common ingredients in curries, sauces, and other cuisine recipes, and are consumed daily by ordinary people (Kubde et al., 2010). Furthermore, Indian traditional herbs, such as betel leaf, have therapeutic and nutritional properties and are used by around 15-20 million Indians (Guha et al., 2006). As a result of their dietary and nutraceutical value, no test extracts should cause any toxicological syndrome in treated individuals at a therapeutic dose.

FGWE and BLWE ameliorate arthritic parameters like paw swelling, arthritis score:

Within 24 hours of receiving CFA, animals showed considerable peripheral edema of the injected paw at the ankle joints. The FGWE (Fenugreek water extract) and BLWE (Bottle gourd water extract) showed considerable anti-inflammatory and anti-arthritic action in proteinase mediated casein breakdown and heat-induced BSA denaturation models, based on in vitro data. As a result, these two extracts were chosen for in vivo anti-arthritis testing. In comparison to the model group rats, dosing of FGWE at 100 and 200 mg/kg p.o. reduced paw edema considerably from day 16 ($p < 0.05$). Dexamethasone (0.75 mg/kg) reduced paw edema in arthritic rats from days 16 to 28. The paw edema in FGWE and BLWE treated animals was dramatically decreased by the 24-28th day of therapy (Table 5.4). The group treated with FGWE and BLWE at 100 and 200 mg/kg showed a substantial decrease in clinical arthritis score from the 15th to the 28th day, which was consistent throughout the treatment period (Table 5.4). The powerful effectiveness of dexamethasone (0.75 mg/kg) was also assessed from

days 15 to 28, and the arthritis score observed by FGWE and BLWE at 200 mg/kg was practically the same **(Table 5.4)**.

Table 5.4: Assessment of paw edema in CFA-induced arthritic model following the treatment of BLWE and FGWE.

Treatment	0th D	4th D	8th D	12th D	16thD	20th D	24th D	28th D
CFA (Disease control)	0.832	1.019	1.062	1.083	1.098	1.133	1.192	1.236
Standard (Dexamethasone 1.5 mg/kg)	0.851	0.898	0.978	0.984	0.898	0.892	0.873	0.534
FGWE 100 mg/ kg	0.855	0.870	0.956	1.066	0.988	0.940	0.900	0.860
FGWE 200 mg/kg	0.860	0.950	1.120	1.022	0.950	0.880	0.846	0.594
BLWE 100 mg/kg	0.833	0.961	1.123	1.231	0.991	0.983	0.901	0.878
BLWE 200 mg/kg	0.854	0.971	1.165	1.114	0.961	0.889	0.861	0.605

At 200 mg/kg, fenugreek and betel leaf fractions effectively protected against CFA-induced arthritic paw edema by 52% and 51% respectively, on day 28. Furthermore, at 200 mg/kg, FGWE and BLWE reduced arthritis index by 86 and 83%, respectively, on the 28th day, which was equivalent to the result (88% decrease of arthritis index) produced by the conventional medicine dexamethasone (0.75 mg/kg). Both extracts significantly reduced arthritis scores in a dose-dependent manner, confirming their promising anti-arthritic potential against RA (Table 4). The arthritic index values obtained on the 28th day were utilised for ANOVA test. In all cases, the p-value was less than 0.001, indicating that the null hypothesis was incorrect, i.e., the samples (FGWE and BLWE) and the standard (dexamethasone) exhibit distinct anti-arthritic efficacy. The standard's F value was 217.4, indicating that the model is significant. The 100 mg/kg and 200 mg/kg FGWEs had F values of 85.7 and 208.3, respectively. The 100 mg/kg and 200 mg/kg BLWEs had F values of 24.1 and 284.1, respectively. As a

result, 200 mg/kg BLWE acted similarly to dexamethasone, followed by 200 mg/kg

FGWE and 100 mg/kg FGWE and BLWE, respectively (Table 5.5).

Table 5.5: Effect of BLWE and FGWE on arthritis score of CFA-induced

arthritic rat model.

Treatment Groups	Arthritic Index (Mean ± S.D)		
	Day 15	**Day 21**	**Day 28**
CFA (Disease control)	3.82±0.112	3.87±0.256	3.91±0.01
Standard (Dexamethasone 0.75 mg/kg)	*1.898±0.085	**0.971±0.080	**0.486±0.056^e
FGWE 100 mg/ kg	2.87±0.020	*1.821±0.014	**0.913±0.023^a
FGWE 200 mg/kg	*2.34±0.012	**1.234±0.021	**0.551±0.015^b
BLWE 100 mg/kg	2.94±0.001	*1.91±0.031	**1.235±0.041^c
BLWE 200 mg/kg	*2.65±0.031	**1.45±0.041	**0.675±0.011^d

Ⅲ **Evaluation of *Euphorbia tirucalli* against arthritis and asthma**

Inflammatory disorders like arthritis and asthma are diseases very much related to each other in respect of the etiological roles of the pro-inflammatory cytokines in disease progression, which is also the overall mediator of pain and inflammation (Tesmer et al., 2008). We wanted to investigate the bioactive fractions derived from roots for the management of pain and inflammation cascade as an organized drug, i.e., having the crude drug material that represents plant parts, because the biopolymeric fractions of the crude latex of *E. tirucalli* (Bani et al., 2007) have been reported to have significant antiarthritic activity, so we wanted to explore the bioactive fractions derived from roots for the management of pain and inflammation cascade as an organized drug. When paw licking, paw edema, and writhing models in mice were compared to the standard medication as a positive control, the total steroid and terpenoid rich fractions produced from hydro-alcoholic root extract (HAETR) revealed a considerable analgesic and anti-inflammatory activity.

The extraction of *Euphorbia tirucalli* root using a cold maceration process in a hydro-alcoholic solvent system (30:70) yielded 12.5 percent. HAETR root extract was found to be high in steroids, terpenoids, and tannins, as well as mild to moderate amounts of alkaloids, saponins, and glycosides, according to phytochemical analysis. TLC analysis of the HAETR extract in the chloroform: methanol (9:1) and chloroform: ethyl acetate: methanol (2:4:4) mobile phases revealed 6 bands with Rf of 0.15, 0.23, 0.67, 0.87, 0.90, 0.97 and 0.16, 0.28, 0.70, 0.90, 0.93, 0.98, respectively. In the chloroform: methanol (9:1) mobile phase, Rf of 0.67 and 0.87 verified the presence of steroid and terpenoid, respectively, as demonstrated by spraying with vanillin–phosphoric acid and p-anisaldehyde spraying reagent (Table 5.6).

Table 5.6: Phytochemical screening and Rf evaluation of HAETR extract by TLC

Phytochemical compound		Hydro-alcoholic root extracts of ET					
		Chloroform: methanol (9:1)			Chloroform: ethyl acetate: methanol (2:4:4)		
		Rf	Spraying reagent	Color after spraying	Rf	Spraying reagent	Color after spraying
Alkaloid	+	0.15			0.16		
Tannin	+++	0.23			0.28		
Flavonoid	-						
Steroid	+++	0.67	Vanillin-phosphoric acid	Pink	0.70	Vanillin-phosphoric acid	Pink
Terpenoid	+++	0.87	p-Aanisaldehyde-sulfuric acid	Red	0.90	p-anisaldehyde-sulfuric acid	Red
Saponin	++	0.90			0.93		
Glycoside	++	0.95			0.98		
Amino Acid	-						

According to phytochemical analysis, our processed STF-HAETR fraction included solely steroids and terpenoids, with no additional secondary metabolites. According to the TLC chromatogram, the separated fraction of STF-HAETR was composed of four bands with Rf values of 0.72, 0.78 (steroid), and 0.91, 0.98 (terpenoid), as shown in Table 5.7. The Rf values obtained from the bioactive fractions of STF-HAETR were 0.78 and 0.91, respectively, when lupeol and beta-sitosterol were used as phytomarkers.

Table 5.7: Phytochemical screening and Rf evaluation of STF-HAETR fraction by TLC

Phytochemical compound		STF-HAETR fraction chloroform: methanol (9:1)		
		Rf	Spraying Agent	Colour
Alkaloid	-			
Tannin	-			
Flavonoid	-			
Steroid	+++	0.72, 0.78	Vanillin-phosphoric acid	Pink
Terpenoid	+++	0.91,0.98	p-Anisaldehyde-sulfuric acid	Red
Saponin	-			
Glycoside	-			

Amino Acid	-			
B-Sitosterol	+++	0.78	Vanillin-phosphoric acid	Pink
Lupiol	+++	0.91	*p*-Anisaldehyde-sulfuric acid	Red

Preliminary HPTLC analysis revealed 11 different peaks in the chromatogram, as shown in Fig. 5.9. The seven significant peaks with Rf: 0.03, 0.12, 0.39–0.46, and 0.62–0.74 were discovered. It also indicated four smaller peaks with Rf values of 0.21, 0.30, 0.52, and 0.56. When we analyzed all of these peaks in a spectrum scan (graphical), we discovered that as the repeat number grew, the peak became clearer and sharper, implying that the drug's unique chemical became more refined/purified. Figures 5.9 a and 5.9b illustrate the results.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	
1	0.02	165.4	0.03	265.5	45.59	0.05	0.0	1357.4	13.32	unknown
2	0.08	0.2	0.12	74.1	12.72	0.18	0.1	3459.7	33.96	unknown
3	0.18	0.0	0.21	11.0	1.88	0.23	2.2	234.6	2.30	unknown
4	0.28	2.9	0.30	14.9	2.55	0.31	9.2	261.2	2.56	unknown
5	0.36	9.6	0.39	25.0	4.30	0.42	8.6	806.5	7.92	unknown
6	0.42	8.6	0.46	25.5	4.38	0.50	15.9	1032.9	10.14	unknown
7	0.51	14.9	0.52	40.4	6.93	0.54	10.2	485.6	4.77	unknown
8	0.54	10.4	0.56	25.6	4.39	0.58	19.7	456.8	4.48	unknown
9	0.60	20.0	0.62	42.0	7.21	0.63	18.0	756.9	7.43	unknown
10	0.68	14.0	0.68	16.1	2.76	0.71	1.1	323.1	3.17	unknown
11	0.72	1.4	0.74	42.3	7.27	0.77	16.8	1013.3	9.95	unknown

Fig. 5.9:   Phytochemical characterization of STF-HAETR by HPTLC chromatogram.

Assessment of *in-vivo* nociceptive assay

The results illustrated in Fig. 5.10 demonstrates STF-HAETR executed a dose-dependent inhibition of the acetic acid-induced abdominal constrictions in mice, demonstrating $35.72 \pm 9.5\%$ protection at 30 mg/kg. Maximal inhibition of $69.37 \pm 2.5\%$ abdominal constrictions was displayed by 60 mg/kg of STF-HAETR. Whereas the intraperitoneal treatment of mice with the positive control, indomethacin at 5 mg/kg, exhibited $70 \pm 1.1\%$ inhibition of writhing compared to untreated controls. The results showed that STF-HAETR provided nearly identical protection against abdominal constriction to the commercially available standard medication (Indomethacin).
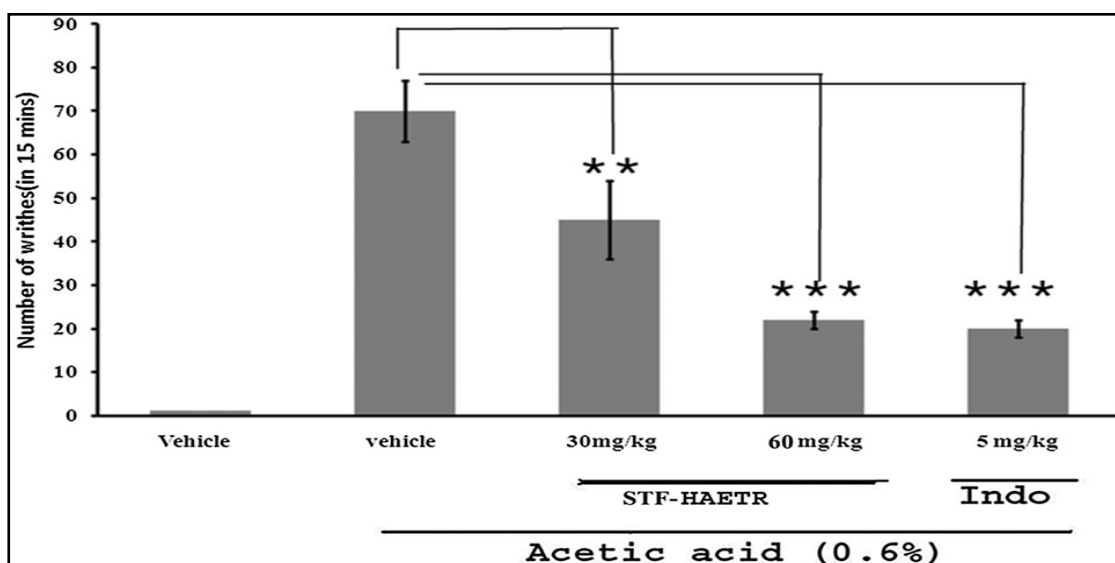
Fig. 5.10: Peripheral analgesic activity compared to standard drug indomethacin.

Furthermore, at 30 and 60 mg/kg body weight, STF-HAETR considerably improved the delay of paw licking time in Eddy's hot plate model, demonstrating central analgesic action, compared to vehicle controls. Maximum protection was determined by the reaction time (92s.) observed with aceclofenac sodium (20 mg/kg) at 60 mg/kg dosages of STF-HAETR, which was equivalent to the reaction time (92s.) obtained with

aceclofenac sodium (20 mg/kg) as shown in Fig. 5.11. The test bioactive fraction provided superior protection against central nociceptive pain than aceclofenac sodium, a common painkiller, according to the findings.
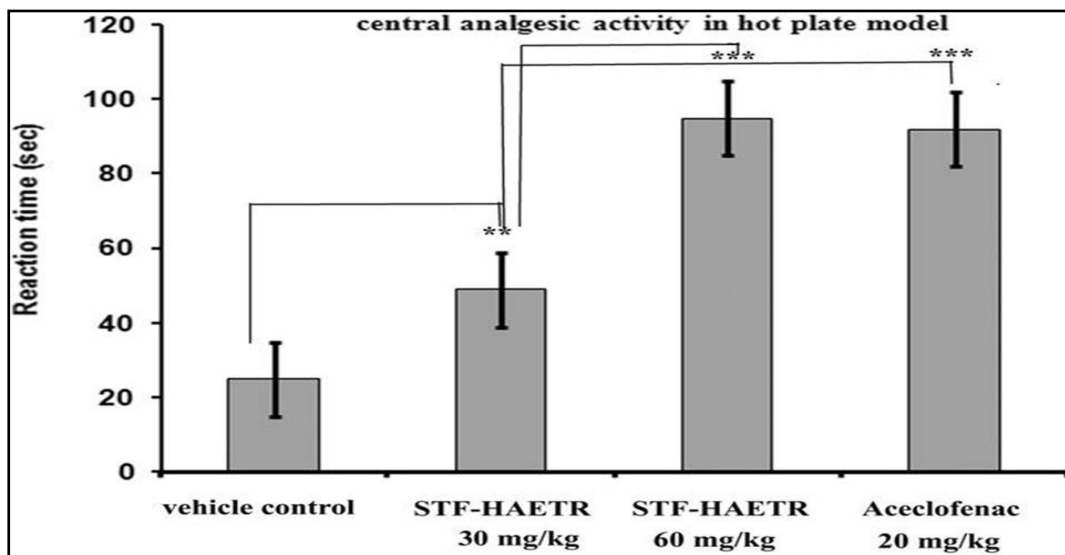
Fig. 5.11: The central analgesic activity of STF-HAETR

The hot plate method of inducing pain is a common paradigm for researching central nociceptive assays because it is considerably more selective for medicines that act centrally (Vale et al., 2004). However, carrageenan, an inflammatory substance, is mostly utilized as a pharmacological tool in rats to assess hyperalgesia caused by inflammation. The anti-inflammatory activity of glucocorticoids, which is very evident in carrageenan-induced paw edema models (Enomoto et al., 2007) with modest effects in other analgesia models, most likely indicates anti-inflammatory activity through an immunosuppressive effect and downregulation of the genes for pro-inflammatory cytokine (Barnes., 1998), COX-2 (Masferrer et al., 1990; Lee et al., 1992).

Anti-inflammatory evaluation

In carrageenan-induced animal models, STF-HAETR extract, reduced edema formation in third hour by 62.37% (P<0.01) (50 mg/ka), %, and 84.42% (P<0.001) (100 mg/kg) in a dose-dependent manner. This result also extended and radically improved up to the fifth hour (P<0.001) exhibiting 72.56 and 96.97% protection towards inflammatory paw edema, respectively. The most interesting finding is that our STF-HAETR extract displayed improved and higher anti-inflammatory potential compared to indomethacin as reference drug which is shown in Fig. 5.12.
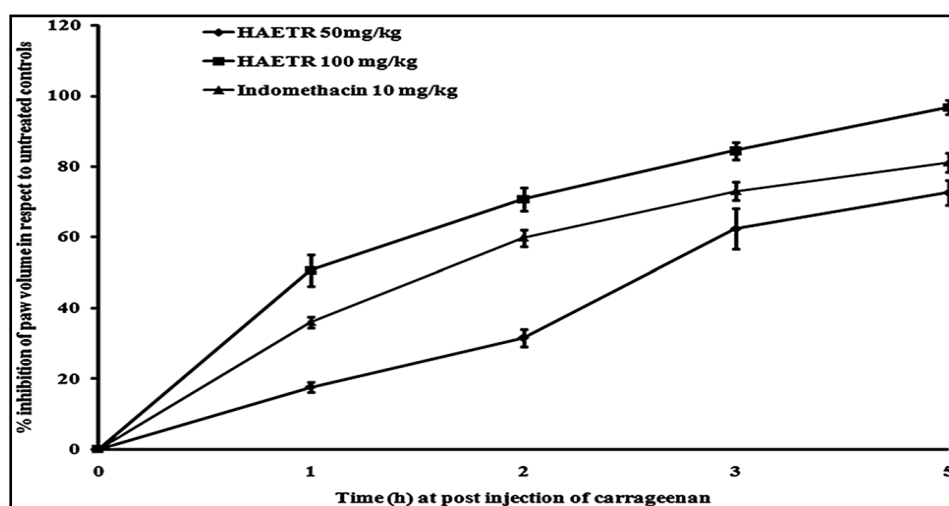
Fig. 5.12: Alleviating effect of STF-HAETR on carrageenan-induced rat paws edema for assessing the acute anti-inflammatory effect.

Effect of plant fraction on LPS-induced NO and proinflammatory cytokine production

Nitric oxide production by LPS-stimulated cells at 10 ng/mL. The inhibition of PBMC cells was shown to be considerable. STF-HAETR in a dose-dependent manner (P0.05). with reductions of 25, 32.5, 70.84, and 80% at 10, 25, 50, and 80 % when compared to untreated controls, the concentrations were 100 µg/mL and 100 µg/mL, respectively.

(Fig. 5.13), indicating that it has anti-inflammatory properties. STF-HAETR treatment of 5 cells resulted in the formation of nitrite being reduced by 8, 21, 68.5, and 76.32%, respectively. In comparison to the positive control, indomethacin is a NO inhibitor in the usual sense.

Human PBMC-derived macrophage cells treated with LPS and CON A showed a substantial up-regulation in the levels of IL-12, TNF-a, and IL-6 in the culture supernatants (Fig. 5.13). STF-HAETR was capable to diminish the levels of all three cytokine levels significantly in a dose-dependent manner. It demonstrated the highest level, i.e., 44 (97.73%) fold reduction (P<0.0001) on LPS CON-A induced IL-6 production at 100 µg/mL compared to untreated controls. Other doses like 10, 25, and 50 µg/mL suppressed 2.26 (P<0.001), 7.33 (P<0.0001), and 22 (P<0.0001) fold of IL-6 production in comparison to untreated controls, respectively (Fig. 5.13a). Vis a vis STFHAETR also reduced TNF-a release by 44.45%.

Similarly, downregulation of the well-known pro-inflammatory cytokine TNF-a by 72.23% (P\0.0001), 61.12% (P<0.0001) was observed on exposure to 100 and 50 lg/ mL of STF-HAETR extract (Fig. 5.13b). STF-HAETR fraction also significantly inhibited IL-12 release by 7.15%, 28.58%, 53.57 (P<0.001) and 64.29% (P<0.0001) at 10, 25, 50 and 100 µg/mL, respectively (Fig. 5.13c).
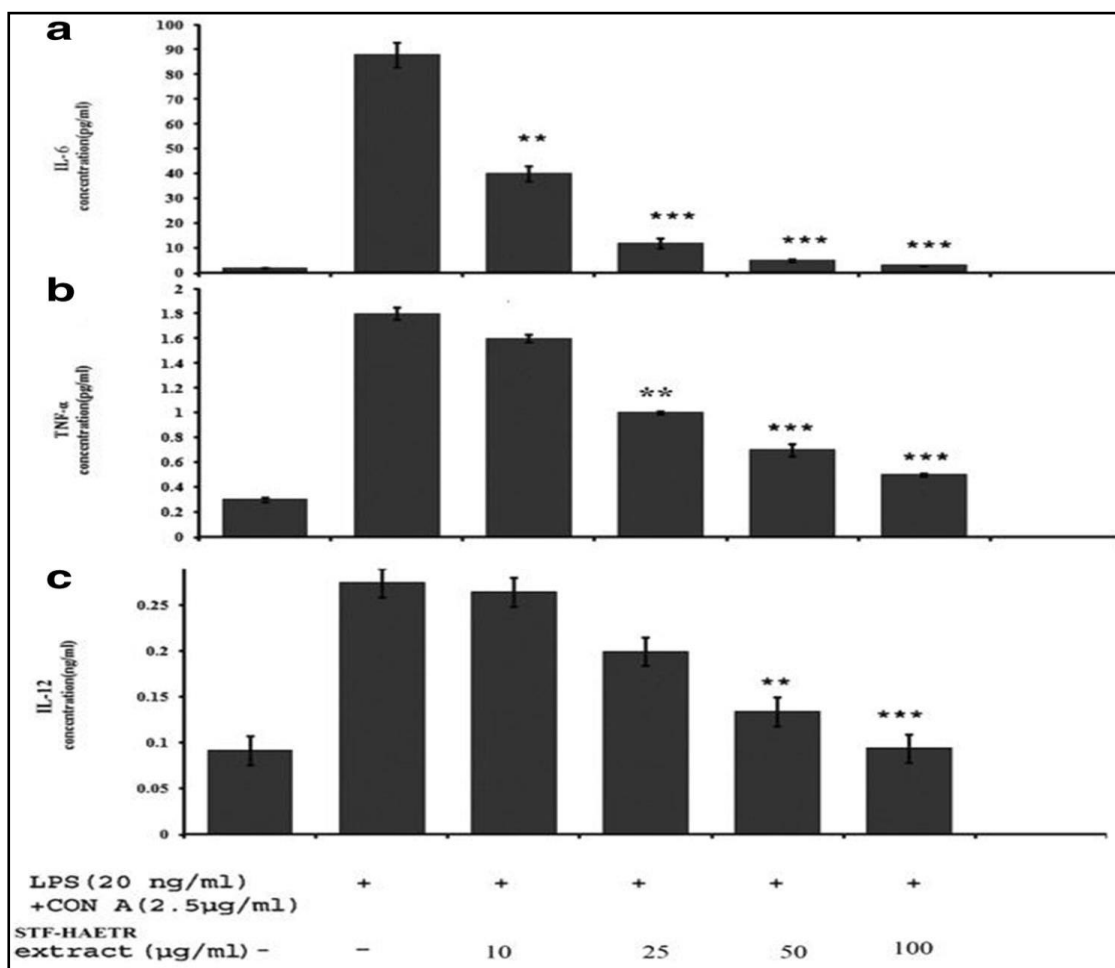
Fig. 5.13: Impact of STF-HAETR on the LPS CON-A induced release of pro-inflammatory cytokines IL-6 (a), TNF-a (b), and IL-12 (c) from PBMC-derived cultured macrophages in a dose-dependent manner.

STF-HAETR decreased the expression levels of the proinflammatory mediator, iNOS:

We used Western blot to look into the impact of the STF-HAETR on iNOS expression. The expression of iNOS was increased after treatment with LPS (1 µg/mL) and IFN ɣ (30 ng/mL) (Fig. 5.14, lane 2). Surprisingly, STF-HAETR inhibited LPS iNOS expression was raised by IFN-c at the IC50 dosage compared to untreated controls (Fig. 5.14, lane 3). The anti-inflammatory impact of STFHAETR, which inhibits i-NOS synthase and hence nitric oxide radical production, was verified in this investigation.
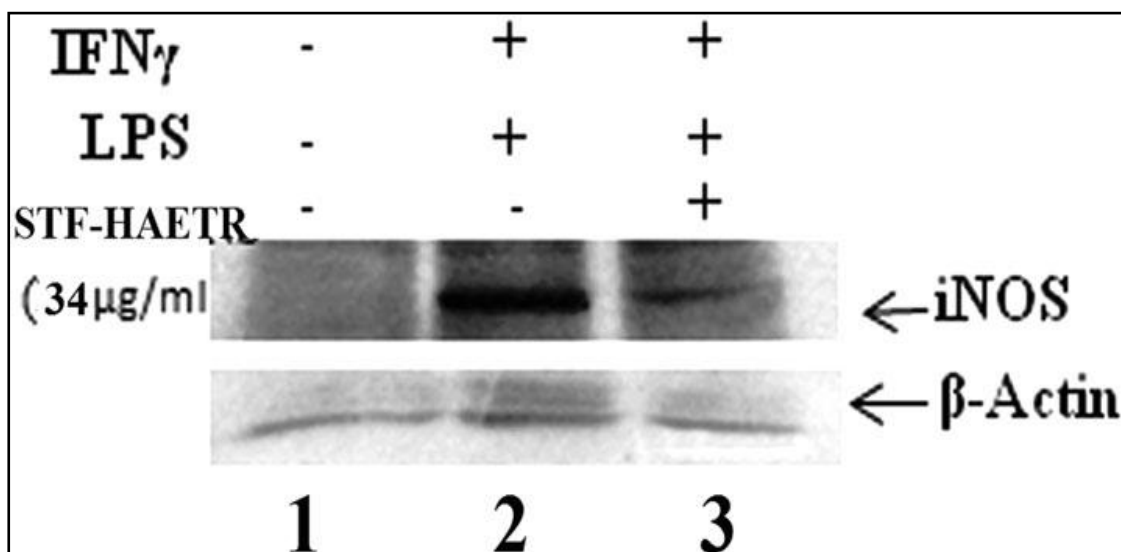
Fig. 5.14 Activity of STF-HAETR on LPS and **IFN-γ induced i-NOS protein expression in RAW264.7 cells by western blot technique**.

Carrageenan-induced inflammation is connected to significant edoema, which is characterised by an elevated level of migration and infiltration of inflammatory polymorphonuclear leukocytes (PMNs) cells, neutrophils, in the inflamed paw tissues, according to several researchers. The in vitro human RBC membrane stabilization action of STF-HAETR against stressors of heat and hypotonicity might be correlated with its ability to stabilize the analogous lysosomal and neutrophil membrane in inflammatory sites, thus preventing the release of lysosomal enzymes and other inflammatory mediators that could have augmented the inflammation. Stabilization

Effects of STF-HAETR extract on COX activity

Purified COX was used as an enzyme source to test the inhibitory effects of STF-HAETR on COX-mediated TMPD oxidation activity. At 100 µg/mL, STF-HAETR did not significantly decrease COX-1 activity. STF-HAETR therapy significantly reduced COX-2 activity, with an IC50 of 25.14 µg/mL. The restraint of the STF-HAETR

measured COX-2 at a much greater level than COX-1. As a result, STF-HAETR is

suggested as a selective COX-2 inhibitor with a selectivity index of 6.55.

Table 5.8: Comparative IC$_{50}$ values (ug/mL) of COX inhibitors

Inhibitors	IC 50		COX-l/COX2 Ratio	Reference
	COX1	COX 2		
TF-HAETR	164.79	25.14	6.55	-
Celecoxib	10.15	0.17	60.0	Tsai et al. (2006)
Diclofenac	0.02	0.1	0.31	Johnson et al. (1995)
Indomethacin	0.001	0.121	0.01	Tsai et al. (2006

As seen in Table 5.8, this outcome was quite similar to celecoxib, a selective COX-2

inhibitor. Figure 5.15 shows the dose-response curve of the test fraction (STF-HAETR)

for COX-1 and 2 inhibition potential.

In comparison to control rats, COX-2 and TNF-a levels in paw edematous rat tissues

increased significantly after carrageenan-induced inflammation, according to previous

study (Lucetti et al., 2010; Nantel et al., 1999). The second phase of paw edema in rats

has similarly been linked to increased levels of NO and COX-2 expression (Posadas et

al., 2004). Carrageenan increased local TNF-a, COX-2, NO, and cytokines production

in the afflicted paws was confirmed in our in vivo paw edema inflammatory model.

COX-2–derived PGs appear to mediate a range of pro-inflammatory effects in this paw

edema model of inflammation, including increased cellular exudation, upregulation of

pro-inflammatory cytokines and COX-2, and so on (Williams and Shacter., 1997). The

previous study has also shown that cytokines, particularly IFN-c, promote COX-2m-

RNA production during macrophage activation in the presence of inflammation when

combined with TNF-a (Arias- Negrete et al., 1995). As a result of the total literature

review, it appears that COX-2 produced prostaglandins and cytokines TNF-a and IFN-c

may have dual vice versa synergistic activating actions in the inflammatory response.
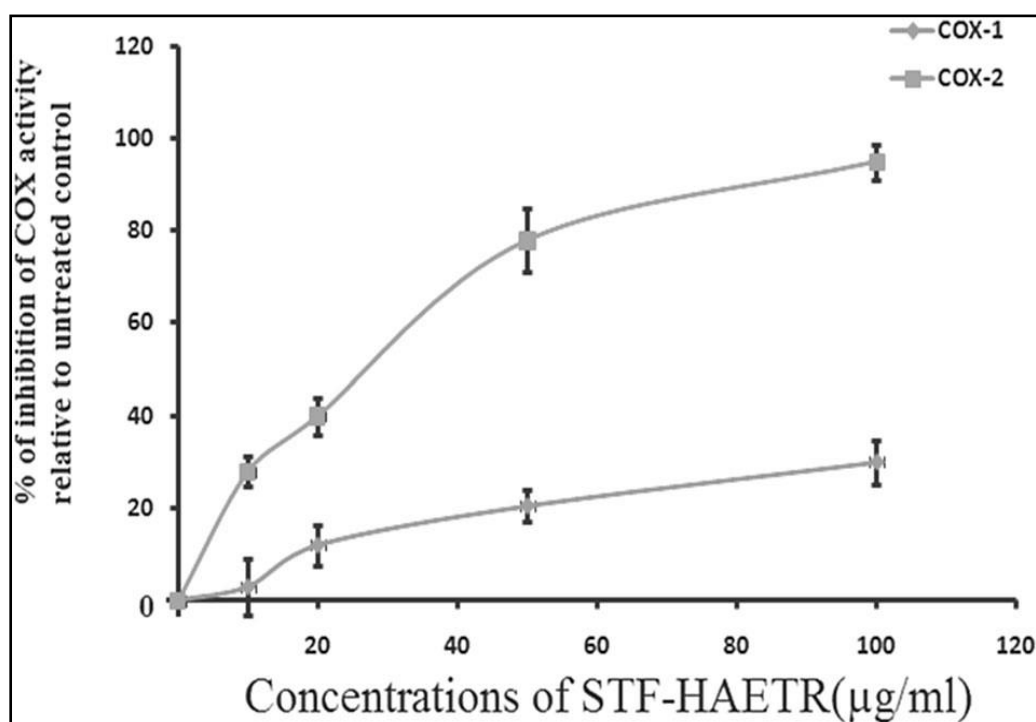
Fig. 5.15: The four different doses (ranging from 10 to 100 lg/ml) have been tested against bovine COX-1 and 2.

***In vitro* anti-asthmatic assessment of STF-HAETR in histamine-induced goat tracheal contraction**

In vitro experiments showed that STF-HAETR decreased histamine-induced bronchoconstriction in isolated goat tracheal tissue preparations. In the absence and presence of STF-HAETR, a dose-response curve was produced. As shown in Table 5.7, STF-HAETR decreased the 0.2 mL histamine solution produced contraction with the highest percentage inhibition (51.37%).

Table 5.9:STF-HAETR effect on protein denaturation, proteinase inhibition, and membrane stabilization

Experiment (g/mL)	STF-HAETR (lg/mL)				Aspirin
	% of protection				
	25	50	75	100	100
% Inhibition of protein denaturation	12.56 ± 0.57	12.56 ± 6.2	53.44 ± 5.45	75.67 ± 3.2	80.86 ± 1.56
% Membrane stabilization	21.56 ± 0.58	42.45 ± 6.2	62.32 ± 1.12	77.51 ± 3.43	65.47 ± 4.21
% Proteinase inhibition	32.23 ± 5.67	46.35 ± 3.45	54.79 ± 5.56	67.47 ± 3.67	62.61 ± 4.45

STF-HAETR effect on protein denaturation, proteinase inhibition, and membrane stabilization

STF-HAETR significantly prevented protein denaturation in the BSA model, with dosages ranging from 25 to 100 µg/mL providing 12.56–75.67% protection. These results were nearly identical to those obtained with the reference medication aspirin at 100 mg/mL. Furthermore, at 100 µg /mL, STF-HAETR stabilized hypotonic saline-induced human RBC membrane lysis by 77.51% making it 1.2 times more efficacious than the conventional medication aspirin at the same dose (Table 5.9). STF-HAETR showed considerable protection against proteinase activity at four distinct graded dosages (25, 50, 75, and 100 µg/mL), with 32.23, 46.35, 54.79, and 67.47% inhibition, respectively. STFHAETR had 1.67 times the anti-proteinase activity of the reference medication (aspirin) at the same dosage of 100 µg/mL.

STF-HAETR significantly reduced in vitro protein denaturation and alleviated trypsin proteinase activity. The anti-denaturation activity of STF-HAETR in the BSA model in vitro shows that protein stabilization may have a therapeutic function during inflammatory reactions like rheumatoid arthritis (Saso et al. 2001). Through protease-activated receptor-2, STF-in HAETR's Vitro trypsin inhibitory capacity can limit

trypsin-mediated activation and release of inflammatory mediators, which is particularly important in osteoarthritis etiology (Miike et al. 2001). Damage to type II collagen on the surface of chondrocytes causes cartilage to degenerate over time in arthritis (Busso et al. 2001). Our preliminary phytochemical analysis of tested root extract part STF-HAETR indicated the presence of significant amounts of mainly two categories of constituents which are steroids and terpenoids. It can be speculated that these ingredients might be highly responsible for the modified immunological effects and lesser toxicity than the earlier reported latex and aerial parts. The anti-inflammatory property of STF-HAETR as shown through its effect on multiple aspects of inflammatory cascades including downregulation of cytokines IL-12, TNF-α, IL-6 and decreased Nitric oxide (NO) formation and potent COX-2 inhibition emphasizes it to be an effective anti-inflammatory drug.

Our main goal was to see if the entire steroid and terpenoid fraction of HAETR might be used as an effective anti-inflammatory with a strong mechanism of action against inflammatory cascades including pro-inflammatory cytokines, COX, and other mediators. As a result, we evaluated the test fraction in a carrageenan paw edema model, which is a well-validated model that causes systemic inflammation with significant increases in proinflammatory cytokines, NO generation, and other factors (Vazquez., 2015). Furthermore, although it mimics the acute inflammatory state, it serves as a classic model for screening anti-arthritics, and NSAIDs, since the etiological factors are quite comparable in contrast to the inflammation associated with osteoarthritis. Models like collagen and CFA caused arthritis, on the other hand, often clarify autoimmune kinds or rheumatoid arthritis, which was not our major goal, but we

will evaluate these models in the future for avoiding chronic inflammatory illnesses like

rheumatoid arthritis.

SUMMARY AND CONCLUSION

The purpose of the first investigation was to examine if *Gelsemium sempervirens* reconstituted tincture might prevent amnesic mice against scopolamine-induced cognitive differences. *In vitro* acetylcholinesterase and secretase enzyme inhibition study, as well as glutathione level assessment in the mouse brain, were used to examine *Gelsemium's* protective mechanism against dementia and azheimer related neurodegeneration. The said study confirmed the attenuated effect of reconstituted *Gelsemium sempervirens* mother tincture on memory impairment, learning dysfunction, and oxidative stress in a scopolamine-induced dementia mouse model, all of which are thought to be symptoms of cholinergic and secretase discrepancy or senile CNS dysfunction in dementia. This study's findings suggest that a low dose of reconstituted mother tincture of *Gelsemium sempervirens* may offer an emerging therapeutic option for the prevention of dementia and related neurodegenerative disorders by improving learning, and memory deficits, and brain oxidative damage in scopolamine-induced amnesic mice. These findings are supported by the combined inhibition of AChE and BACE1 as well as the upregulation of the endogenous antioxidant (GSH) defense mechanism. Gelsemium had no discernible adverse effects or mutagenicity in mice at the experimental therapeutic dosage (data not shown). As a result, Gelsemium may be a good option for medication to combat dementia and related neurodegenerative illnesses viz. Alzheimer disease. However, more research is required to investigate the particular polar active ingredients or bioactive fractions of Gelsemium tincture that are responsible for its anti-dementia efficacy, as well as the likely underlying mechanism.

The second study evaluated the potential of bioactive extracts of *Trigonella foenum-graecum*, *Piper betel*, and *Lagenaria siceraria* homogenate was tested against

arthritis and related inflammation. Fenugreek and betel leaf extracts showed promising anti-inflammatory and anti-arthritic potential in this research work. The anti-arthritis mechanism of extract was thought to be due to the significant inhibition and reduction of TNF-∞ and IL-6. Fenugreek and betel leaf extract significantly reduce cartilage degeneration and collagen protein denaturation in arthritic syndrome caused by pro-inflammatory cytokines. Bottle gourd and betel leaf extracts, on the other hand, demonstrated a strong anti-asthmatic impact by inhibiting the lipoxygenase enzyme and reducing the formation of broncho-constrictor Leukotrienes. The presence of flavonoids, lignin, and mucilage-like phyto components in fenugreek and betel leaf aqueous extract revealed remarkable anti-arthritic potential. The ability of test extracts to lower TNF-∞ and IL-6 levels, and the intensities of COX-2 and 15-LOX enzymes, is thought to be responsible for their anti-arthritic and anti-inflammatory properties and managing the pain. Aqueous extract of Fenugreek seeds and betel leaves showed *in vivo* anti-rheumatoid arthritis action. Furthermore, the asthmatic condition is reduced by bottle gourd homogenate and betel leaf aqueous extract. Because of its promising anti-oxidant efficiency, betel leaf extract exhibited anti-arthritic and anti-asthmatic potential. The current study investigates the causes of these bio-products favorable health benefits (fenugreek, betel leaf, and bottle gourd). As a result, as validated by previous research, these bioactive extracts may be emphasized as a promising anti-inflammatory herbal medicine shortly for the therapy of diverse auto-immune disorders such as rheumatoid arthritis and allergic asthma. Because the doses examined were made up of bioactive phytoconstituents and other nutraceuticals, they were quite safe. Because these herbal vegetables are consumed by common people as traditional nutraceutical based edible dietary foods.

The anti-inflammatory, analgesic, anti-asthmatic, and anti-arthritic properties of the total steroid and terpenoid rich bioactive fractions of the hydro-alcoholic extract of E. tirucalli root were investigated in the third study .According to the findings, the bioactive fraction of STF-HAETR decreased pain by selective COX-2 inhibition and attenuated all types of inflammatory syndromes by downregulating inflammatory NO production by lowering iNOS expression, TNF-α, IL-6, and IL-12 cytokine levels. The anti-inflammatory and anti-nociceptive activities of carrageenan were confirmed in an *in-vivo* model of carrageenan-induced acute inflammatory paw edema, as well as acetic acid-induced peripheral and hot plate-induced central nociceptive pain model. It dilated histamine-induced bronchoconstrictions *in vitro*, that could alleviate asthmatic consequences. Proteinase inhibition by STF-HAETR greatly improved arthritic condition by preserving protein denaturation. As a result, the powerful anti-inflammatory properties of total steroid–terpenoid enriched fractions of *Euphorbia tirucalli* might be investigated as a viable therapeutic candidate in many inflammatory and immunological dysregulation and disorders such as asthma, arthritis, and nociceptive pain via immune modulation.

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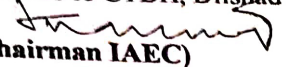
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APPROVAL CERTIFICATE

This is certify that the project title "......*Pharmacological Survey ..... Euphorbia sps.*" ".." has been approved (Approval No: MC/UCMS/IAEC/.........[to be filled by CAH]) by the IAEC, University College of Medical Sciences & GTBH, Dilshad Garden, Delhi – 110 095).

(Chairman IAEC)   4/5/12

(Dr. Subrata Chakraborty)

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(*NOTE: Make sure that minutes of the meeting duly signed by all the IAEC members are maintained by the Office*)

May 2012

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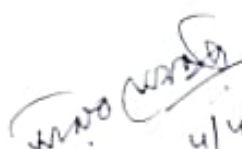
Dated: 04-04-2023

To Whom It May Concern

This is to certify that Dr. Partha Palit, Mr. Dhrubajyoti Mukherjee, *et al.* had undertaken a project titled "Pharmacological Screening of some Herbal Extract in Animal Model" in the Academic Year 2012-2013 at our institution for which, they had takendue animal ethical permission from the Institutional Animal Ethics Committee (IAEC) of Dr. B. C. Roy College of Pharmacy and Allied Health Sciences, Durgapur, West Bengal, India – 713206 (Registration Number: 1348/e/10/CPCSEA dated 06.05.2010).

4/4/2023

(Sagar Sengupta)

Registrar

REGISTRAR
Dr. B. C. Roy College of Pharmacy and
Bidhannagar, Durgapur

Research Paper

Reconstituted mother tinctures of *Gelsemium sempervirens* L. improve memory and cognitive impairment in mice scopolamine-induced dementia model

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A B S T R A C T

Ethnopharmacological relevance: Gelsemium sempervirens (L.) J.St.-Hil is a herb used for the treatment of various neuroses in both homeopathic and Ayurvedic systems. The present study examines whether *Gelsemium* reconstituted tincture can protect against scopolamine induced cognitive discrepancies in amnesic mouse model. In order to investigate the protective mechanism of *Gelsemium* against dementia, *in vitro* acetyl cholinesterase and β-secretase enzyme inhibition and estimation of glutathione level in mouse brain were carried out.
Materials and methods: The inhibition study on acetyl cholinesterase and β-secretase enzyme was conducted on brain homogenate supernatant spectrophotometrically using specific substrate. Cognitive enhancement activity was assessed by elevated plus maze and passive avoidance study in scopolamine induced dementia mouse model. Glutathione, an anti-oxidant, was measured spectrophotometrically from scopolamine induced amnesic mice brain supernatant using 5,5′-dithiobis 2-nitrobenzoic acid in the presence and absence of *Gelsemium* tincture.
Results: Significant inhibition was found with *Gelsemium* on AChE and β-secretase enzyme with an IC_{50} of 9.25 and 16.25 μg/ml, respectively, followed by increasing glutathione levels in comparison to the untreated dementia group. The effect of *Gelsemium* of scopolamine-induced cognitive deficits was determined by measuring the behavioral parameters and the antioxidant status of the brain after scopolamine (1 mg/kg i.p.) injected amnesic mice. *Gelsemium* significantly demonstrated *in vivo* anti-dementia activity (60% protection) and increased exploratory behavior.
Conclusion: Our investigations indicated that alkaloid, iridoids and coumarin enriched reconstituted *Gelsemium* tincture extract displays promising cognitive enhancement in adult mice after short-term oral treatment. Hence, *Gelsemium* can be a promising anti-dementia agent, mediating the protection against amnesia, attention disorders and learning dysfunctions through dual inhibition of both acetyl cholinesterases (no false positive effect was shown), β-secretase and antioxidant activity.

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1. Introduction

Cognitive impairment, amnesia, attention deficit disorders related symptoms caused by psychoneurotic or Neuro-psychiatric diseases or Alzheimer's disease (AD) are a major global health problem. Dementia and related AD are the second most occurring field globally next to cancer (www.alz.org).

The most familiar type of dementia is Alzheimer's disease (AD), although the primary cause of AD and other cognitive impairment remains unclear. Global investigation for understanding the fundamental

pathological mechanisms of such disorders is still going on. The dementia and related disorders are pathophysiologically exemplified by progressive neurodegeneration involving two types of anomalous deposits found in AD brains. They are neurofibrillary tangles (NFT) and extracellular amyloid plaques composed of hyperphosphorylated tau forming paired helical filaments (PHF) and toxic neuro inflammation caused by amyloid β (Aβ) peptide (Ballatore et al., 2007; Knobloch et al., 2007; Laferla et al., 2007). Aβ is produced from b-amyloid precursor protein (b-APP) through sequential cleavage by membrane proteases of b- and c-secretases. The b-secretase 1 (BACE 1) first cuts APP to produce a C-terminal fragment (b-CTF), an immediate substrate for c-secretase, which further cleaves b-CTF to yield Aβ (Willem et al., 2009). Since mutation in b-APP induces the accumulation of Ab1–42 that leads to familial AD related dementia forms (Hardy and Selkoe, 2002). Aβ accumulation has been recommended to play a key role in the etiology of AD related disorders. The loss of the cholinergic neuronal signal and transmission leads to an impairment of higher cortical functions, which is directly related to the progressive deterioration of memory, attention, and cognitive processes (Chopra et al., 2011). In fact, reduction in the acetylcholine concentration in the cholinergic synaptic cleft is observed in the brain of Alzheimer's and as well as dementia patients (Mufson et al., 2008).

Current status of therapeutic management of dementia related disorders is not satisfactory, as it has no permanent cure with existing medication (Francis et al., 1999; Figueiró et al., 2011). Drug therapies against dementia or AD have faced numerous challenges. Many drug candidates derived from Pharmaceutical Industry held promise at one stage of clinical research, but failed at the next phase due to resistance, adverse effect, and poor efficacy in a wide range of patients (Hampel, 2012). However, donepezil, galantamine, rivastigmine, and memantine are the Food, Drug and Administration (FDA) recommended drugs of choice for treatments for AD and dementia, but they are only effective against mild to moderate symptoms of dementia related AD and slow the progression of the disease (www.alz.org). They are restricted towards the severe and later stage of dementia therapy. Therefore, the proper development of nootropic candidate with multiple drug targets for treatment of dementia, cognitive dysfunction and related neurodegeneration would be highly desirable.

Earlier scientific reports and Homeopathic Materia Medica suggested that *Gelsemium sempervirens* (L.) J.St.-Hil (Gelsemiaceae) (a promising neuroprotective candidate); common name Carolina-jasmine or yellow jessamine(in English) can be used to remedy a variety of neurological and behavioral symptoms, including general prostration, drowsiness, tiredness, mental apathy, lack of muscular coordination, anxiety, depression and phobic discomfort associated with unfamiliar situations, aggravated by the emotion and excitement (Barbancey, 1987). *Gelsemium sempervirens* demonstrated sedative, analgesic and anti-seizure properties (Peredery and Persinger, 2004; Dutt et al., 2010; Gahlot et al., 2011) and as per Duke Phytochemical and Ethnobotanical Databases at high pharmacological doses. *Gelsemium sempervirens* is also found to be effective as an anxiolytic agent at ultra-low dose. Homeopathic dilutions of *Gelsemium sempervirens* improved stress-induced behavioral disorders of mice in the staircase and light–dark (LD) tests as validated by recent reports (Bousta et al., 2008). Moreover, *Gelsemium sempervirens* tincture extract inhibited dopamine, noradrenaline and serotonin uptake into synaptosomal preparations from different parts of the rat brain at higher concentration (Cueilleron et al., 1982). Previous reports suggested that three new oxindole alkaloids, 19-(R)- hydroxydihydrogelsevirine, 19-(R)-acetyldihydrogelsevirine and 19-(R)-hydroxydihydrogelsemine, together with the known alkaloids such as 19-(S)- hydroxydihydrogelsevirine, gelsevirine and gelsemine (Long-Ze et al., 1991) and another new oxindole alkaloids, 14β-hydroxygelsedine (Schun and Cordell, 1985) were isolated from this plants. 9-hydroxy substituted iridoids (Søren et al., 1987) and six new indole alkaloids (Kogure et al., 2005) were reported

from *Gelsemium*, too. Principal alkaloid gelsemine from the plant exhibits powerful antinociception in chronic pain (Zhang et al., 2013). Furthermore, a new cytotoxic pregnane steroidal derivative 12 beta-hydroxy-5 alpha-pregn-16-ene-3,20-dione had also been reported from *Gelsemium* (Schun and Cordell, 1987). In addition, bio active coumarin derivatives, scopoletin and scopolin were reported from this plant (Zhang et al., 2008; Bhattacharyya et al., 2009). Principle bioactive compounds of this plant are gelsemine, sempervirine and gelsemicine (Demarque et al., 1995), which increased the hypertensive action of adrenaline (Raymond, 1937).

In connection to above cited versatile findings of *Gelsemium sempervirens* on nervous system and related disorders, we were encouraged to investigate whether the reconstituted mother tincture of *Gelsemium sempervirens* could protect the scopolamine induced amnesia and cognitive dysfunction in animals. The aim of the present study was to assess the role of *Gelsemium* on acetyl cholinesterase (AChE) and β-secretase (BACE1) enzyme inhibition to explore the protective mechanism demonstrated by it against dementia, too.

2. Materials and methods

2.1. Experimental animals

Male swiss albino mice (4-weeks-old), weighing 25–30 g (approved by Institutional animal research ethics committee Ref. no. BCRCP/IAEC/5/2012) were used for experimental purposes. The animals were kept in polyacrylic cages ($38 \times 23 \times 10$ cm^3) with five animals per cage and maintained under standard laboratory conditions (room temperature 24–27 °C and humidity 60–65%). Food in the form of dry pellets and water were available *ad libitum*. The mice were adapted to the laboratory conditions five days before the experimental session. All animal experiments were carried out in accordance with the NIH Guidelines for Care and Use of Laboratory Animals. The Institutional Animal Ethical Committee of Dr. B. C. Roy College of Pharmacy and A.H.S., formed under the Committee for purpose of Control and Management of Experiments on Animals, approved the Pharmacologic protocols.

2.2. Compounds or drugs

Sealed pack hydro-alcoholic (57–61% alcohol) mother-tincture of *Gelsemium sempervirens* (bought from SBL Laboratories Ltd., Uttarakhand, India, Batch number: SB/08/2012 and Voucher number: 24569) was used for the study. All other chemicals such as piracetam (98% pure as HPLC grade, procured from Sigma), scopolamine hydrochloride (HPLC grade, 90% pure purchased from Sigma-Aldrich), galanthamine hydrobromide (TLC grade, 94% pure purchased from Sigma-Aldrich), used for experiments, were of analytical grade.

2.3. Acute oral toxicity study

The acute oral toxicity study was performed as per Organization for Economic Co-operation and Development (OECD) test guidelines (Test no., 425, 2008) following Up-and-Down procedure.

2.4. Experimental protocol

Hydro-alcoholic mother tincture (57–61% alcohol) of *Gelsemium sempervirens* was purchased from homeopathic medicine shop, manufactured by SBL Laboratories Ltd., Uttarakhand, India, Batch number: SB/08/2012; Registration number: 1/UA/HPM/2006. Phytochemical composition of procured tincture was well characterized and manufactured as per Indian homeopathic pharmacopoeia,

Appendix – XXXIII and French homeopathic pharmacopoeia, 2002, ANSM general monographs. It was used as reconstituted lyophilized powder redissolving in water for injection (WFI) immediately to study the anti-dementia activity at a dose of 1 mg/kg orally on a daily basis for consecutive 14 days. The dose was selected on the basis of 1/10th of LD_{50} doses, carried out as per OECD and World Health Organization (WHO) guidelines. Mice were divided into five groups, containing 6 animals in each group for the evaluation of cognitive measurements in respective exteroceptive behavioral models.

Treatment protocols are described schematically in the following table:

recorded on the first day (day 14). If the animal did not enter into one of the covered arms within 90 s, it was gently pushed into one of the two covered arms and the TL was assigned as 90 s The mouse was allowed to explore the maze for 10 s and then returned to its home cage. Memory retention was examined on the next day (day 15) by again recording the TL.

2.5.2. Passive shock avoidance paradigm

Passive avoidance behavior based on negative reinforcement was recorded to examine long-term memory (Jeong et al., 2008). The apparatus consisted of a box ($27 \times 27 \times 27$ cm^3) having three

Group	I, vehicle control	II, untreated control	III, *Gelsemium* treated group (without scopolamine)	IV, *Gelsemium* treated group (with scopolamine)	V, positive control (standard group)
No. of mice	6	6	6	6	6
Treatment	WFI orally for 14 days	WFI orally for 14 days+scopolamine 1 mg/kg IP on 14th day after 60 min of last dose of WFI	Reconstituted and lyophilized *Gelsemium* powder dissolved in WFI 1 mg/kg orally for 14 days	Reconstituted & lyophilized *Gelsemium* powder dissolved in WFI 1 mg/kg orally for 14 days+scopolamine 1 mg/kg IP on 14th day after 60 min of last dose of *Gelsemium*	Piracetam 200 mg/kg IP from 8th to 14th day+scopolamine 1 mg/kg IP on 14th day after 60 min of last dose of piracetam
Procedure	Behavioral test performed after 90 min of last WFI dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant	Behavioral test performed after 30 min of scopolamine dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant	Behavioral test performed after 90 min of last *Gelsemium* dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant	Behavioral test performed after 30 min of scopolamine dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant	Behavioral test performed after 30 min of scopolamine dose ↓ Brain dissected ↓ Homogenized ↓ Centrifuged ↓ Enzyme assay with supernatant

2.5. Cognitive measurements (exteroceptive behavioral models)

2.5.1. Elevated plus maze (EPM)

The elevated plus maze was served as the exteroceptive behavioral model (wherein the stimulus existed outside the body) to evaluate learning and memory in mice. This model was performed as per established method adopted with slight modification (Kulkarni and Mahesh, 2011). The apparatus consisted of two open arms (16 cm $\times$ 5 cm) and two covered arms (16 cm $\times$ 5 cm $\times$ 12 cm). With the arms extended from a central platform (5 cm $\times$ 5 cm), a maze was elevated to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was taken as the time taken by the mouse to move into any one of the covered arms with all its four legs. TL was

wooden walls and one Plexiglas wall, featuring a grid floor (3 mm stainless steel rods set 8 mm apart), with a wooden platform ($10 \times 7 \times 1.7$ cm^3) in the center of the grid floor. The box was illuminated with a 15 W bulb during the experimental period. Electric shock (20 V AC) was delivered to the grid floor. Training was carried out in two similar sessions. Each mouse was gently placed on the wooden platform set in the center of the grid floor. When the mouse stepped down and placed all its paws on the grid floor, shocks were delivered for 15 s and the step-down latency (SDL) was recorded. SDL was defined as the time taken by the mouse to step down from the wooden platform to the grid floor with its entire paw. Animals showing SDL in the range (2–15 s) during the first test were used for the second session and the retention test. The second-session was carried out 90 min after the first test. When the animals stepped down before 60 s, electric

shocks were delivered for 15 s. During the second test, the animals were removed from shock free zone if they did not step down for a period of 60 s. Retention was tested after 24 h in a similar manner, except that the electric shocks were not applied to the grid floor. Each mouse was again placed on the platform, and the SDL was recorded with an upper cut off time of 300 s.

2.6. Estimation of biochemical parameters

All the biochemical estimations were performed at the end of behavioral test as follows.

2.6.1. Brain tissue preparation

Whole brains of each treated and untreated mice were dissected after completion of behavioral studies, respectively at 15th day and cleaned with ice cold saline in a petri dish kept on ice. Tissues were weighed and homogenized in an ultra turrax T-25 homogenizer at 9500 rpm with 0.03 M sodium phosphate buffer (pH-7.4) to make 10% w/v homogenate and were used for subsequent estimations. Similarly, in order to assess the *in vitro* acetylcholine esterase activity, whole brain homogenates were prepared as per above cited method from the normal healthy mice.

2.6.2. Estimation of protein in the brain samples

Protein was estimated in all the supernatant of brain samples by Bradford's reagent using bovine serum albumin (BSA) as standard in the range of 0.01–0.1 mg/ml (Bradford, 1976).

2.6.3. Estimation of in vitro brain acetyl cholinesterase (AChE) activity

The brain homogenate was centrifuged at 100,000g at 4 °C in a Beckman Ultracentrifuge (LE 80, USA), using a fixed angle rotor (80 Ti) for 60 min. Supernatant was collected and stored at 4 °C for acetyl cholinesterase estimation by Ellman's method with slight modification (Ellman et al., 1961). The effect of *Gelsemium sempervirens* crude hydro-alcoholic tincture on the activity of supernatant containing AChE was studied *in vitro* at different concentrations (2–75 μg/ ml) with the final reaction volume of 3.14 ml. 20 μl of aliquots of tincture solution was incubated for 5 min at 37 °C containing 0.4 ml aliquots of the supernatant (source of AChE enzyme) and was added to a cuvette containing 2.6 ml phosphate buffer (0.1 M, pH 8) and 100 μl of 5,5′-dithiobis (2-nitrobenzoic acid (DTNB 98% pure, as the TLC grade from Sigma-Aldrich)). The reaction was then initiated by the addition of 20 μl of acetylthiocholineiodide ((ATCI); 99% pure, as the TLC grade from Sigma-Aldrich)), a substrate of AChE enzyme. The end product, yellow 2-nitro-5-sulfidobenzene-carboxylate anion was measured spectrophotometrically at a wavelength of 412 nm as formed by the reaction of DTNB with thiocholine released by the enzymatic hydrolysis of acetylthiocholineiodide for 10 min. The percentage inhibition of cholinesterase activity was calculated using the following formula (Ahmed and Urooj, 2010):

Inhibition of AChE(%) $= (\delta A \, \text{control} - \delta A \, \text{sample}/\delta A \, \text{control})/\delta A \, \text{control} \times 100$

2.6.4. In vitro BACE1 (β-secretase) enzyme assay

The assay was performed according to protocol with slight modifications (Jeon et al., 2003). Briefly, a mixture of 10 μl of assay buffer (50 mM sodium acetate, pH 4.5), 10 μl of BACE1 (1.0 U/ml; Sigma-Aldrich), 10 μl of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate (sigma)), and 10 μl of reconstituted mother tincture (2–75 μg/ml) dissolved in 0.2% dimethyl sulphoxide (DMSO) was incubated for 60 min at room temperature in the dark. The fluorescence of each sample was monitored at exciting wave length of 540 nm and the emission intensity at 580 nm. The percentage of inhibition was estimated by the following equation:

Inhibition % $= [1 - \{(S - S_0) /(C - C_0)\}] \times 100$, where C is the fluorescence of the untreated control (enzyme, buffer, and substrate containing 2% DMSO) after 60 min of incubation, C_0 is the fluorescence of control at zero time, S is the fluorescence of the tested samples (enzyme, sample solution, and substrate) after incubation, and S_0 is the fluorescence of the tested samples at zero time. IC_{50} value was estimated using Graph Pad Prism 4.0 (Graph Pad Software Inc., USA) through regression analysis.

2.6.5. Estimation of brain acetyl cholinesterase activity in vivo

Drug treated and untreated supernatant (0.4 ml aliquot) samples derived from brain homogenate were added to a cuvette containing 2.6 ml phosphate buffer (0.1 M, pH 8) and 100 μl of DTNB (Raju et al., 2004). The contents of the cuvette were mixed thoroughly and absorbance was measured at 412 nm using a UV–visible spectrophotometer (Shimadzu, USA). It was recorded as the basal reading when absorbance reached a stable value and then 20 μl acetylthiocholine iodide was added as substrate. Change in absorbance was recorded for a period of 10 min at 30 s intervals. One unit of acetyl cholinesterase activity was defined as the number of micromoles of acetylthiocholine iodide hydrolyzed per min per mg of protein. The specific activity of acetyl cholinesterase is expressed in μmol/min/mg protein (Raju et al., 2004).

2.6.6. Estimation of brain reduced glutathione (GSH) levels

GSH was determined by its reaction with DTNB to yield a yellow chromophore which was measured spectrophotometrically. The brain homogenate was mixed with an equal amount of 10% TCA and centrifuged at 2000g for 10 min at 4 °C, which was used for GSH estimation. 2 ml of phosphate buffer (pH 8.4), 0.5 ml of DTNB and 0.4 ml of double-distilled water were added into 0.1 mol of processed tissue sample and the mixture was shaken vigorously on vortex. The absorbance was monitored at 412 nm using the UV–visible spectrophotometer (Shimadzu, USA). GSH was calculated by using standard curve prepared with reduced glutathione and expressed as μg/mg protein (Tota et al., 2011).

2.7. TLC bioautographic assay with reconstituted Gelsemium tincture through true TLC based Ellmans's method and thiocholine and DTNB inhibition reaction

The reconstituted tincture of *Gelsemium* was dissolved in methanol and water (1:1 v/v) to obtain a solution of 20 μg/ml. Drug loaded TLC plate (stationary phase: silica gel) was developed for chromatogram in a mobile phase of chloroform and methanol (9:1, v/v) and 2% antimony trichloride in 10% methanolic KOH as spray reagent. The plate was completely dried and saturated by spraying with 5 mm ATCI, DTNB (dissolved in 50 mM of Tris HCl buffer, pH 8 and 37 °C) and dried. Then 3 U/mol of AChE (50 mM of Tris HCl buffer, pH 8 and 37 °C) was sprayed on to the plate which generated yellow colored background with white spots which is AChE inhibiting extracts monitored after 5 min according to Ellman's method. With the intention to check whether the positive results revealed by the reconstituted *Gelsemium* tincture in the TLC or the microplate assay were due to true enzyme inhibition or due to inhibition of the chemical reaction between DTNB and thiocholine, the false positive reactions were also recognized as per the method reported earlier (Rhee et al., 2003). Another plate was developed and sprayed with 5 mm of DTNB (50 mM of Tris HCl buffer, pH 8, 37 °C) and after drying, it was sprayed with 5 mm of ATCI and 3 U/mL of AChE dissolved in 50 mM of Tris–HCl buffer, pH 8, the appearance of white spots on a yellow background resembles false-positive results. Galanthamine (10 μg/ml) was used as a positive control of true AChE inhibitor in both cases of the experiment along with tested *Gelsemium* extracts. White
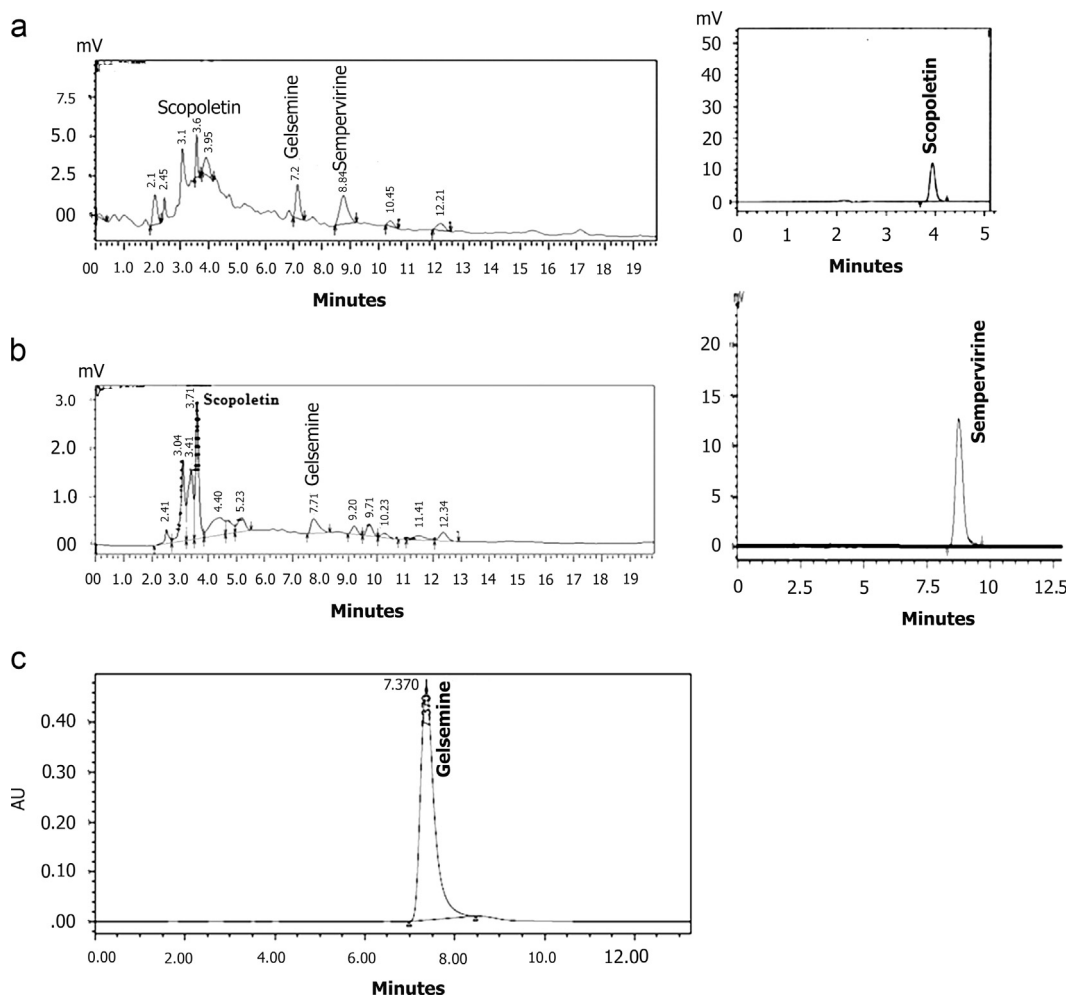
Fig.1. HPLC characterization of mother tincture of *Gelsemium sempervirens* in (a) methanol–water mobile phase (40:60 in 0.1% trifluoro acetic acid), showing 9 major peaks representing active phytoconstituents including gelsemine at retention time of 7.20, validated by chromatogram of pure gelsemine (shown in c). (b) Acetonitrile–water mobile phase (40:60 in 0.1% trifluoro acetic acid), showing 12 peaks representing active phytoconstituents. (c) HPLC chromatographic peak of gelsemine (procured from sigma-Aldrich) after injection of 500 µg/ml, in reverse phase chromatographic system using methanol–water mobile phase (40:60 in 0.1% trifluoro acetic acid), eluting at retention time of 7.28 min. The numerical value at the top of each peak is signifying retention time of phytocompound. HPLC chromatogram is carried out in Shimadzu HPLC at reverse phase chromatographic system (specification: column- C-18 column; length and width − 250×4.60 mm^2; run time 20 min; flow rate1 ml/min at λ_{max} 258 nm wavelength in isocratic system).

spots on a yellow background were recorded and compared with each other in both the cases. The results obtained from the spectrophotometric assessment were compared with the results of the TLC assay (Fig. 5).

2.8. Acetyl cholinesterase mRNA expression by reverse transcription polymerase chain reaction (PCR)

RNA was isolated from treated and untreated mice brain using TRIzol reagent (Sigma) as directed by the manufacturers. Concentration and purity of RNA were determined spectrophotometrically using Gene Quant. Approximately 2 µg of total RNA was reverse transcribed using reverse transcriptase (RT) in a 20-µl mixture containing oligo-(dT)-primer, RNase inhibitor, dNTP mix and 5X reaction buffer (Omniscript RT kit). The resultant cDNA was amplified separately with specific primer for AChE using Taq PCR core Kit (Qiagen USA). Briefly, cDNA was amplified in a 20 µl reaction volume containing 1 U Taq polymerase, 200 µM (each) dNTP mix and 2 µl 10X Taq buffer with specific primers. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Bioer XP cycler) through 35 cycles at the specifications described as follows:

Primer AChE 5′-GATCCCTCGCTGAACTACACC-3′ Annealing temperature 60 °C. 5′-GGTTCTTCCAGTGCACCATGTAGGAG-3′, Product size 331 bp (Tota et al., 2011).

2.9. Statistical analysis

All the results were expressed as mean ± standard error (SEM). All values were statistically analyzed using unpaired student *t* test and one way analysis of variance (ANOVA) followed by Turkey's post-hoc analysis. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Chemical profile of major phyto-components present in the Gelsemium mother tincture

Alkaloids: Gelsemine (8% w/w), and sempervirine (1.6% w/w)
Coumarin: Scopoletin (1.67% w/w), and scopolin (0.8% w/w)
Iridoid: Gelsemide (1.31% w/w)
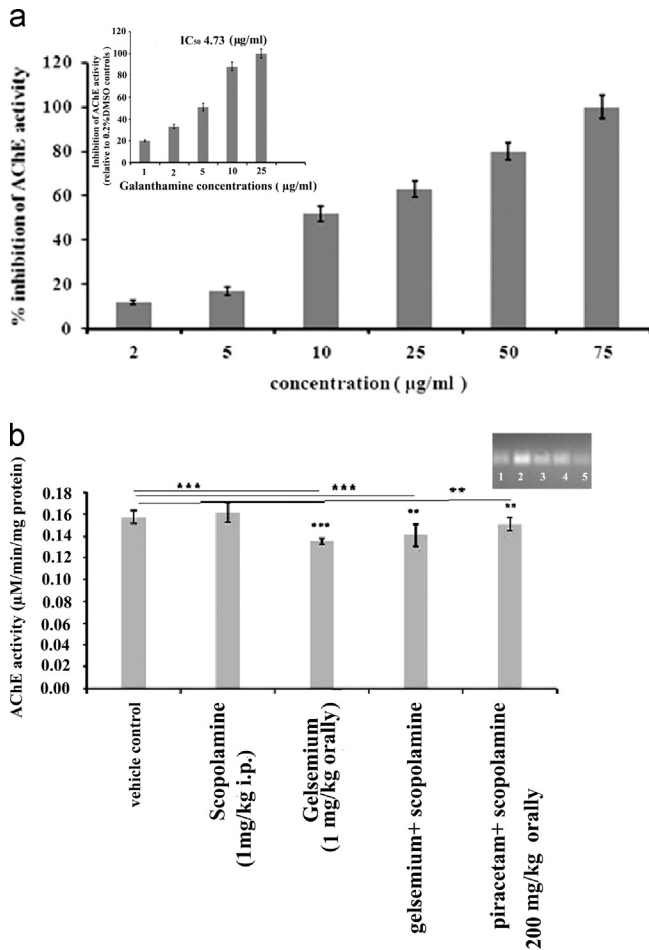
Fig. 2. The efficacy of reconstituted mother tincture of *Gelsemium sempervirens* on (a) *in vitro* inhibition of AChE activity in mice brain at concentrations dependent. Observations were compared to the results of galanthamine, a standard inhibitor of AChE, shown in inset. The results are expressed as mean $\pm$ S.E.M ($n=3$), representative of three similar experiments. (b) Effect of reconstituted *Gelsemium sempervirens* mother tincture treatment on *in vivo* AChE activity in the brain of scopolamine treated amnesic mice. $***P < 0.0001$, $**P < 0.001$, considered as significant difference when compared to scopolamine treated controls, assessed by two tailed unpaired student t test. Data are expressed as the mean $\pm$ SEM ($n=6$ mice per group), representative of two similar experiments. Reconstituted mother tincture of *Gelsemium sempervirens* significantly reduced the AChE mRNA expression in scopolamine treated mice, which is shown in inset. RT-PCR based AChE mRNA expression of lane 1: untreated vehicle controls; lane 2: only scopolamine treated; lane 3: *Gelsemium* pre-treatment in scopolamine treated; lane 4: piracetam (standard, positive control) pre-treatment in scopolamine treated; and lane 5: only *Gelsemium* treated, centrifuged sup from mice brain homogenate, demonstrated at inset of Fig. 2b.

Fig. 6 shows the structures of the above-cited compounds. Percentage of chemical profile in the mother tincture was assessed according to French homeopathic pharmacopoeia, 2002, ANSM general monographs and Søren et al. (1987).

3.2. Effect of reconstituted Gelsemium tincture on ex vivo acetylcholinestrase (AChE) activity

Inhibition study of *in vitro* acetylcholine esterase illustrated that reconstituted *Gelsemium sempervirens* tincture remarkably inhibited AChE derived from brain tissue homogenate in a dose dependent manner in comparison to untreated controls as presented in Fig. 2a. It showed 100% AChE inhibition at 75 μg/ml with an IC$_{50}$ of 9.5 μg/ml compared to untreated controls ($P < 0.001$), while galanthamine, a standard inhibitor demonstrated IC$_{50}$ of 4.73 μg/ml on *in vitro* AChE inhibition (Fig. 2a; inset).
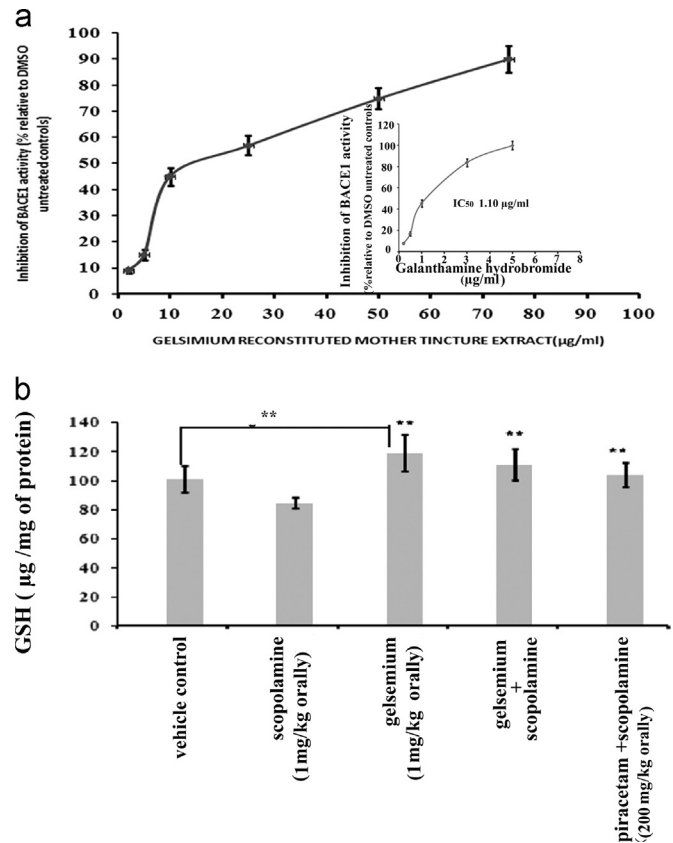
Fig. 3. Effect of reconstituted *Gelsemium sempervirens* mother tincture treatment on (a) the inhibitory activity of BACE1 in a concentration dependent manner. Impact of galanthamine, a standard inhibitor for BACE1, as positive control on the inhibitory activity of beta secretase in a concentration dependent manner was represented at inset. The results are expressed as mean $\pm$ S.E.M ($n=3$), representative of three similar experiments. (b) The level of GSH in the brain of scopolamine treated amnesic mice. Data are expressed as mean $\pm$ S.E.M. ($n=6$ mice per group), representative of two similar experiments. $**P < 0.001$, considered as significant difference when compared with untreated controls and vehicle controls, assessed by two tailed unpaired student t test.

3.3. In vivo AChE inhibition study at post-treatment of Gelsemium

Short term (14 days) treatment with *Gelsemium* (1 mg/kg. p.o.) resulted in a significant reduction of specific AChE activity [$F (1,10)=12$, $P < 0.01$] in comparison to scopolamine treated dementia mice. *Gelsemium* treated group showed better *in vivo* AChE inhibition in comparison to standard nootropic drug piracetam treated mice group [$F (1,10)=4.8$, $P < 0.05$]. *Gelsemium* showed better performance in comparison to standard memory enhancer drugs, piracetam, showing *in vivo* AChE inhibition even at 200 times lesser dose. These were further confirmed by evaluation of the mRNA expression level in mouse brain, where mRNA expression of AChE was significantly increased in only scopolamine injected mice group (Fig. 2b inset; lane 2). But *Gelsemium* pre-treatment in scopolamine injected mice profoundly decreased mRNA expression level of AChE in comparison to only scopolamine treated dementia mice (Fig. 2b inset; lane 3). *Gelsemium* treated group also demonstrated significant inhibition of AChE mRNA expression level in comparison to the positive controls (piracetam) treated mice brain homogenate (Fig. 2b inset; lane 4).

3.4. True- and false-positive activity assessment by Gelsemium through TLC bioautographic assay

The reconstituted *Gelsemium* tincture that showed the most promising activity by enzyme assay spectrophotometrically was

tested for the inhibitory activity of AChE by TLC bioautographic assay and confirmed whether the reaction is due to true inhibition by the enzymes or not. All the polar compounds separated by TLC chromatogram were found to be the true AChE inhibitor supported by the TLC assay (Fig. 5a; lane 1) and comparable with galanthamine, a standard positive control inhibitor of AChE (Fig. 5a; lane 2). No false positive reactions were observed; instead white spots of inhibition were monitored for the potent active _Gelsemium_ tincture extract (Fig. 5b; lane 1) and galanthamine (Fig. 5b; lane 2). The results achieved from the TLC based true AChE inhibition study was similar to the outcome of spectrophotometric enzyme inhibition assay of AChE. 2% antimony trichloride in 10% methanolic KOH was used as spraying reagents to identify the polar compounds from _Gelsemium_ tincture extract (Fig. 5c; lane 1).

3.5. In vitro BACE1 inhibition

Gelsemium demonstrated 90% inhibition of BACE1 activity at 75 µg/ml compared to untreated controls ($P < 0.001$), in a concentration dependent manner with an IC_{50} of 16.25 µg/ml (Fig. 3a). Our findings were comparable with those of a standard positive control drug, galanthamine (IC_{50} of 1.10 µg/ml), and an inhibitor of β-secretase (Fig. 3a; inset).

3.6. Estimation of reduced glutathione level in mouse brain

With the aim of investigating the impact of _Gelsemium sempervirens_ tincture on brain oxidative status, we measured the reduced glutathione level at post-treatment of _Gelsemium_ in scopolamine induced dementia mice. As observed in Fig.3b, the levels of reduced glutathione significantly increased ($\sim 31\%$) in the brain tissue homogenate after _Gelsemium_ treatment (1 mg/kg, p.o. in which the jasmine was present as 0.08 mg/kg (data not shown) for 14 days) in comparison to control mice [F (1,10)=73.973, $P < 0.001$]. Interestingly, GSH content is higher in _Gelsemium_ treated mice compared to piracetam treated scopolamine induced dementia mice [F (1,10)=4.138, $P=0.069$]. _Gelsemium_ treatment significantly [F (1,10)=13.407, $P=0.004$] enhanced the GSH level as compared to vehicle controls.

3.7. Effect of reconstituted Gelsemium tincture on behavioral models

3.7.1. Elevated plus maze study

In order to investigate the protective role of _Gelsemium_ on scopolamine induced memory impairment, we conducted behavioral studies measuring the TL (second) of mice in elevated plus maze model. Results demonstrated that after 14 days treatment with the reconstituted _Gelsemium sempervirens_ mother tincture at 1 mg/kg, the tested mice regained its loss of memory function of almost 60% [F (1,10)= 100.38, $P < 0.001$] against scopolamine induced dementia, while standard nootropic drug piracetam (200 mg/kg i.p.) showed 70% [F (1,10)= 132.165, $P < 0.001$] learning improvement over untreated scopolamine induced amnesic group (Fig. 4). Transfer latency on the 14th day of _Gelsemium_ treatment revealed progress in learning behavior of animals, while transfer latency of next day reflected retention of information or memory, decreasing transfer latency time compared to 14 day observation. Both _Gelsemium_ (1 mg/kg, oral) and piracetam (200 mg/kg, i.p.) significantly [F (2,15)=108.956, $P < 0.001$] reduced the transfer latency time in comparison to scopolamine treated memory impaired mice.

3.7.2. Passive avoidance study

In order to evaluate the improvement of dementia mediated loss of memory and learning disorders at post therapy of _Gelsemium_, the passive avoidance test through step down latency was carried out in
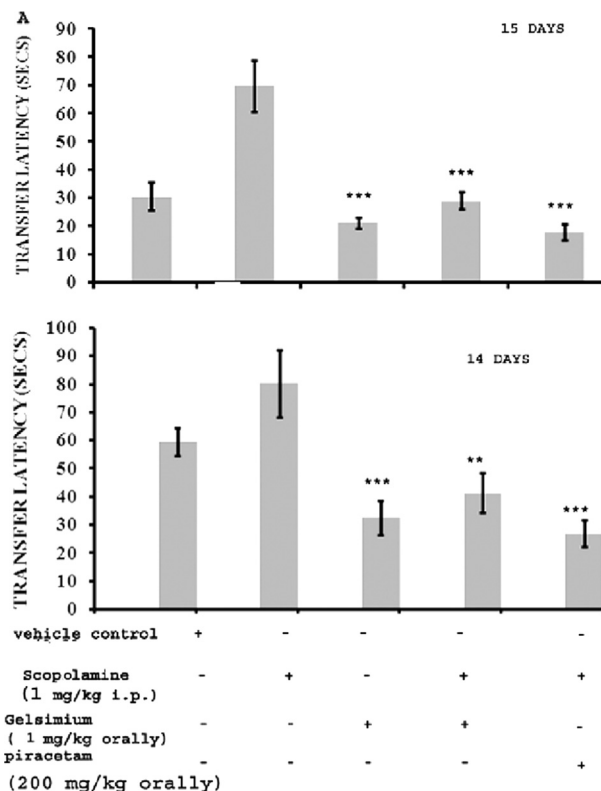
Fig. 4. Presentation of the anti-dementia effect of reconstituted mother tincture of _Gelsemium sempervirens_ on transfer latency time(s) in elevated plus maze transfer latency test, ***$P < 0.0001$, **$P < 0.001$, considered as significant difference when compared with untreated controls _i.e._ scopolamine (1 mg/kg i.p.) treated group, assessed by ANOVA followed by two tailed unpaired student t test. The results are expressed as mean ± S.E.M. ($n=6$ mice per group), representative of two similar experiments.

scopolamine induced amnesic mouse model. The step-down latency time (s) of the scopolamine-treated mice was significantly shorter compared to vehicle control mice [F (1,10)=138.922, $P < 0.001$]. After _Gelsemium_ (1 mg/kg p.o.) treatment for 14 days in scopolamine-treated mice, step-down latency was considerably enhanced on day 15 (Table 1), compared to untreated controls [F (1,10)=145.494, $P < 0.001$]. This learning improvement activity exhibited by _Gelsemium_ was almost comparable to standard nootropic drug, piracetam. Moreover, the important findings of our experiment were that step-down latency time of only _Gelsemium_ treated mice group was superior to vehicle treated mice group in the passive avoidance test. The findings revealed the memory enhancing activity of _Gelsemium_.

4. Discussions

The present study explored the attenuated effect of reconstituted _Gelsemium sempervirens_ mother tincture on memory impairment, learning dysfunction, and oxidative stress on scopolamine induced dementia mouse model, that are considered to be attributes of cholinergic and β-secretase discrepancy or senile CNS dysfunction in dementia (Drever et al., 2007). _Gelsemium_ improved acetylcholine neurotransmission in hippocampus by inhibiting the AChE enzyme, decreased BACE1 activity and up-regulate the GSH content to protect dementia mice against cognitive and memory impairment.

Previous reports suggested that hippocampal AChE has the modulating role in cognitive performance. It is a critical component for the dementia-related memory deficits and underlying process of

Table 1
Effect of reconstituted *Gelsemium sempervirens* mother tincture on scopolamine induced PA retention deficits in mice, measuring step-down latency (s).

	Vehicle controls	Copolamine (1 mg/kg i.p.)	Gelsemium (1 mg/kg orally)	Gelsemium+scopolamine	Piracetam (200 mg/kg i.p.)+scopolamine
Step down latency (s)	41.16 ± 3.5	17 ± 3.6	58 ± 85[#]	43.5 ± 4[***]	71.5 ± 9[***]

[***] $P < 0.001$, considered as significant difference when compared with untreated controls *i.e.* scopolamine (1 mg/kg i.p.) treated group, assessed by ANOVA followed by two tailed unpaired student *t* test.
[#] $p < 0.0001$, considered as significant difference against vehicle controls. The results are expressed as mean ± S.E.M. (n=6 mice per group), representative of two similar experiments.

cognitive disorders (Das et al., 2005). Therefore, inhibition of AChE is an attractive strategy for the treatment of dementia and associated cognitive disorders (Terry and Buccafusco, 2003; Trinh et al., 2003). Our study also validated the earlier concepts regarding the management of dementia demonstrating *in vitro* reduction as well as promising *in vivo* inhibition of AChE activity by *Gelsemium*. Our results suggest that a correlation between *in vitro* and *in vivo* AChE inhibition exists. It may also enhance the cholinergic activity, raising the brain acetylcholine level in cholinergic synapses (Lane et al., 2006), and thereby ameliorate the cognitive and memory dysfunctions in the dementia related neurodegenerative disease. Our findings of *Gelsemium* induced AChE inhibition are the true enzyme inhibition due to polar compounds present in the chromatogram, not demonstrated false positive effect in TLC based inhibition of the chemical reaction between thiocholine and DTNB. Alkaloid, iridoid, and coumarin like compounds exhibited in the polar region of the chromatogram are seems to be responsible for eliciting the true enzyme inhibition. However, we have assessed the mRNA expression level of AChE inhibition by the *Gelsemium* that does not suffer from the problem associated with false-positive effect due to carbonyl, aldehyde or amine function containing compounds that interfere with the color reaction in Ellman's reagent. From this consequence, the polar fraction would be chosen for the further isolation of specific AChE inhibitors as exposed by 2% antimony trichloride in 10% methanolic KOH spraying reagents (Fig. 5c; lane 1).

Scopolamine, a muscarinic receptor antagonist, crosses the blood brain barrier and induces dementia and cognitive dysfunction, resulting in deficits in the learning, acquisition, and short-term retention of spatial memory tasks by blocking cholinergic neurotransmission and reducing hipocampal volume during dementia (Yamada et al., 2008). Hence, reconstituted *Gelsemium sempervirens* mother-tincture could be used successfully for the therapeutic management of the AChE induced intervention of cholinergic transmission related to dementia. Supporting results showed suppression of mRNA expression of AChE (Tota et al., 2011) at molecular level due to *Gelsemium* pre-treatment, implying that it specifically may enhance the memory molecule, *i.e.* acetylcholine at postsynaptic region to recover the dementia disorder associated with neurodegenerative diseases. Our inhibition study on AchE mRNA level was further confirmed by the enzyme inhibition of AChE activity by the *Gelsemium* treatment in both *in vitro* and *in vivo* at protein level. Our finding has been validated by earlier reports too (Tota et al., 2011; Ozarowski et al., 2013). Therefore we can conclude that inhibition of AchE activity has been revealed and correlated to both genetic and protein levels to establish the mechanism of anti-dementia property elicited by *Gelsemium*. Moreover, AChE activation could be linked to the augmentation of lipid per oxidation followed by producing free radicals and extensive oxidative stress (Kaizer et al., 2005; Liu et al., 2009; George et al., 2013). Hence *Gelsemium sempervirens* mother tincture contains iridoid and coumarin compounds, as supported by our phytochemical test (Table 2) and earlier reports (Chaudhuri et al., 1999; Kitajima et al., 2003; Jeong et al., 2008). These compounds might be responsible for restoring the levels of reduced GSH, both in the cortex and hippocampus, due to their free radical scavenging activity (Shinomol et al., 2013).
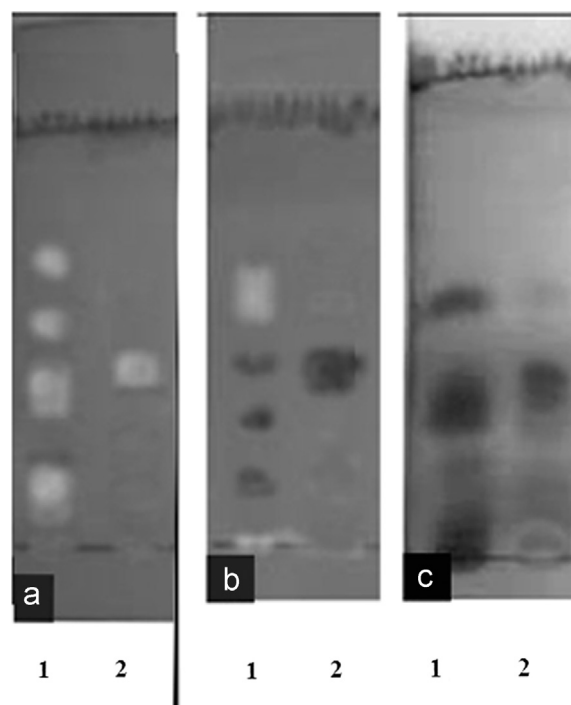
Fig. 5. The presentation of true and false positive inhibition of acetylcholine esterase by reconstituted *Gelsemium* tincture extract. The silica gel TLC layer was loaded with lane 1: *Gelsemium* extract (20 μg/ml) lane 2: galanthamine (10 μg/ml). The mobile was taken as choloroform: methanol (9:1). (a) White spots in yellow background correspond true enzyme inhibition while 5 mM ATCI and DTNB (dissolved in 50 mM of Tris HCl buffer, pH 8 and 37 °C) sprayed on to the TLC chromatogram layer and followed by 3 U/mL of AChE (50 mM of Tris HCl buffer, pH 8 and 37 °C). (b) White spots in yellow background represent the false positive inhibition when the layer was sprayed with 5 mM ATCI and DTNB, dissolved in 50 mM of Tris HCl buffer, pH 8 and 37 °C on to the TLC chromatogram layer. (c) The similar chromatogram was sprayed with 2% antimony trichloride in 10% methanolic KOH reagent in order to display the true AChE inhibiting polar compounds.

Furthermore, *Gelsemium* inhibited remarkably BACE1, a crucial enzyme in Aβ formation and subsequently prevented the formation of senile plaques and tangles from mutated amyloid beta precursor protein (Bejar et al., 1999; Scarpini et al., 2003; Sastre et al., 2008). These findings implied that it might be used for elimination of the pathophysiological symptoms of AD related to cognitive disorders and Aβ induced neurotoxicity, dementia (Parihar and Hemnani, 2004; Hardy and Selkoe, 2002; Willem et al., 2009).

Hence, dual inhibition of AChE and β-secretase enzyme activity (Lane et al., 2006) could be effective and powerful treatment strategy for learning disturbances, memory loss and other psycho-behavioral disorders. *Gelsemium* displayed an important anti-oxidant activity, increasing the GSH content in brain mice. The study revealed that reconstituted *Gelsemium sempervirens* tincture could be used for repairing the memory and cognitive disorders produced due to free radical generation and broad oxidative stress induced by scopolamine. It may also prevent the Aβ plaque formation induced by activated BACE1 and subsequent GSH depletion occurred during

Table 2
Phytochemical investigations of mother tincture of Gelsemium sempervirens, and their R_f evaluation through thin layer chromatography.

Phytochemical compound		_Gelsemium sempervirens_ mother tincture, chloroform:methanol:acetic acid:water (6:2:1:1)		
		R_f	Spraying reagent	Color
Alkaloid reagent	+++	0.36	Dragendorff's	Orange
Tannin	−			
Flavonoid	−			
Steroid	+++	0.78	Vanillin-phosphoric acid	Pink
Terpenoid	+	0.88	p-Anisaldehyde-sulfuric acid	Red
Iridoids	+++	0.54	Antimony chloride	Violet brown
Coumarin	+++	0.60	10% methanolic KOH	Yellowish-green
Saponin	−			
Glycoside	−			
Amino acid	−			

(+++) appreciable amount; (++) moderate amount; (+) trace amount; (−) completely absent
phytochemical screenings in the purchased mother tincture of _Gelsemium sempervirens_ were performed as per TLC method using mobile phase of choloroform:methanol (9:1) and followed by spraying with specific chemical reagents on chromatographic band after drying of plates. Precise color development/changes into relevant R_f band after heating of TLC plate indicates the presence of exact phytochemical category in mother tincture.

pathogenesis of neurodegenerative diseases (El-Sherbiny et al., 2003; Jung et al., 2010).

The experimental results of elevated plus maze and passive avoidance tasks confirm the amelioration of amnesia, improvement of learning skills and cognitive impairment in mouse model induced by _Gelsemium_. Only 1 mg/kg of reconstituted _Gelsemium_ tincture fed for 14 days significantly restored scopolamine induced impairment of spatial memory tasks in both exteroceptive behavioral models, whereas similar type of cognitive enhancing activity was obtained from standard memory enhancers such as _Bacopa monnieri_ and _Ginko biloba_ plant extract at 30 and 60 mg/kg, respectively (Das et al., 2002). The behavioral studies suggested that _Gelsemium_ treated dementia mice achieved an outstanding retention capacity of memory and learning function. This is the first report so far demonstrating enhancement of learning, memory and cognitive skills by _Gelsemium_ on the amelioration of dementia related neurodegenerative diseases and it is consistent with previous studies (Jung et al., 2010; Aremu et al., 2011; Papandreou et al., 2011). Dual inhibition of AChE and BACE1 might be the plausible mechanism of action for _Gelsemium_ to provide neuroprotection against dementia. _Gelsemium sempervirens_ mother tincture is a hydro-ethanolic solution (57–61%) consisting of strychnine like indole alkaloids such as gelsemine, sempervirine, uvaol, and 2-(4-hydroxyphenyl) ethyl heptadecanoate, coumarin (scopoletin, and scopolin), iridoids, as supported by our present phytochemical investigations, carried out according to the standard method (Paech and Tracy, 1955; Harbourne and Harborne, 1998). Among them, strychnine like gelsemine alkaloids and iridoid compounds might be responsible for exhibition of such dual inhibition as approved by earlier reports (Zhang et al., 2008) (Fig. 6).

10 μg/ml reconstituted tincture of _Gelsemium sempervirens_ contained 0.8 μg/ml pure gelsemine (an oxi-indole alkaloid derivative, HPLC retention time 7.3 min, depicted in Fig. 1) as a major ingredient in the purchased tincture, estimated and characterized by standard curve of pure gelsemine based on AU and matching of retention time from HPLC chromatogram with the 98% pure gelsemine procured from sigma. In addition, two other bioactive agents such as scopoletin (a coumarin derivative; retention time 3.8 min) and sempervirine (indole alkaloid; retention time 8.8 min) were characterized in the HPLC chromatogram (Fig. 1) from the standard compounds, gifted by IICB, Kolkata. Herein, we carried out phytochemical screening with the purchased tincture to validate the presence of different phytochemical category as reported earlier in Homeopathic pharmacopoeial literature. Other indole derivatives like sempervirine, _etc._ are present in the tincture as reported and verified in Homeopathic pharmacopoeia (Indian homeopathic pharmacopoeia, Appendix – XXXIII and French homeopathic pharmacopoeia,
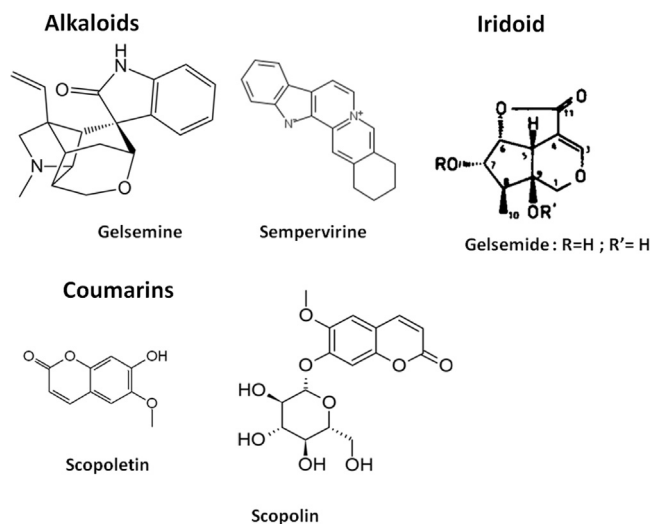
Fig. 6. Structures of major bioactive compounds like alkaloids, coumarins and iridoid present in the _Gelsemium_ mother tincture.

2002, ANSM general monographs). Gelsemine contains similar functional groups like strychnine and increases the synthesis of 3α, 5α-tetrahydroprogesterone or allopregnenolone (3α, 5α-THP) neurosteroid (Kitajima et al., 2003), that might be responsible for memory and cognitive enhancement and improved acetyl cholinergic networks (Venard et al., 2008; Frye, 2009).

Anti-dementia activity was marvelously enhanced by _Gelsemium_ treatment may be due to the dual mode of drug targeting and synergistic action of indole like derivatives and scopoletin, scopolin, a coumarin derivatives as anti-Alzheimer agents (Patil et al., 2013), present in this crude mother tincture.

Neurodegenerative disease related dementia, cognitive impairment and other mental aberrations not only affects aged people but also younger people even at the ages between 15 and 35 yr, which hampers daily life performances (Darnaudéry et al., 2002; Pennington et al., 2003; Raina et al., 2008). Therefore _Gelsemium_ therapy could be beneficial for them.

Gelsemium sempervirens was found to be effective in the treatment of anxiety, stress, phobia and depression as mother tincture and Homeopathic medicine, as supported by previous scientific reports and traditional use (Felter and Lloyd, 1983; Magnani et al., 2010). Therefore _Gelsemium_ treatment may help people to recover from dementia, who often suffer from significant levels of psychoneurotic disorders during the progressive stage of the disease

(www.med.nyu.edu/adc/forpatients/ad.html; www.everydayhealth.com/alzheimers/alzheimers-depression-and-anxiety.aspx).

Since reconstituted *Gelsemium sempervirens* tincture is found to be effective as BACE1 inhibitor to prevent the Aβ accumulation induced tangles and plagues formation in AD, so it needs to be evaluated in transgenic mice to reveal its antiAlzheimer activity. Therefore still further investigations should be required in molecular aspect.

5. Conclusion

These collective results conclude that low dose of reconstituted mother tincture of *Gelsemium sempervirens* may offer an emerging therapeutic option for the prevention of dementia and related neurodegenerative disorders by improving learning, memory deficits and brain oxidative damage in scopolamine induced amnesic mouse model. Those findings are supported by dual inhibition of AChE and BACE1 and up regulation of endogenous antioxidant (GSH) defense system. *Gelsemium* displayed no apparent side-effect and mutagenicity at the experimental therapeutic dose in mice (data not shown). Therefore, *Gelsemium* can be a promising candidate of pharmacotherapy to defend against dementia and associated neurodegenerative diseases.

However, further investigations are required to identify the specific polar active constituents or bioactive fractions of the *Gelsemium* tincture responsible for its anti-dementia activity and the possible underlying mechanism.

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Inflammopharmacology

ORIGINAL ARTICLE

Attenuation of nociceptive pain and inflammatory disorders by total steroid and terpenoid fraction of *Euphorbia tirucalli* Linn root in experimental in vitro and in vivo model

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Abstract The plant *Euphorbia tirucalli* Linn has been successfully used as a tribal folk medicine in India and Africa for the management of acute inflammatory, arthritic, nociceptive pain and asthmatic symptoms. The present study was conducted to assess the anti-inflammatory, analgesic, anti-asthmatic and anti-arthritic role of the total steroid and terpenoid rich fractions of the hydro-alcoholic extract of *E. tirucalli* root (STF-HAETR). STF-HAETR fraction demonstrated 71.25 ± 2.5 and $74.25 \pm 5.1\%$ protection against acetic acid-induced pain and central neuropathic pain at 75 and 100 mg/kg doses, respectively. It showed 96.97% protection against acute inflammation at 100 mg/kg with 1.6-fold better activity than the standard drug. The fraction exhibited such efficacy via inhibition of proinflammatory cytokines TNF-α, IFN-γ, by 61.12 and 65.18%, respectively, at 100 μg/mL. Inhibition of cyclooxygenase and Nitric oxide synthase in a dose-dependent manner affirms its analgesic and anti-inflammatory activity. The spectrophotometric analysis reveals that STF-HAETR induces ameliorative effect against heat-induced denaturation of Bovine serum albumin (BSA) and exhibits significant anti-proteinase activity. The plant fraction also demonstrated anti-asthmatic activity by displaying 62.45% protection against histamine induced bronchoconstriction or dyspnoea. Our findings suggest that STF-HAETR could be an effective safe therapeutic agent to treat nociceptive pain, acute inflammation, asthma, and arthritis which may authenticate its traditional use.

Abbreviations

ET	*Euphorbia tirucalli*
ERK	Extracellular signal-regulated kinases
IL	Interleukin
IFN	Interferon
MIP	Macrophage inflammatory protein
PK	Protein kinase
COX	Cyclooxygenase
NSAIDs	Nonsteroidal anti-inflammatory drugs
iNOS	Inducible nitric oxide synthase
TNF-α	Tumor necrosis factor-α
IL-12	Interleukine-12
STF-HAETR	Total steroid and terpenoid rich fractions of hydro-alcoholic extract of *Euphorbia tirucalli* root
TLC	Thin layer chromatography

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HPTLC	Hi-performance thin layer chromatography
LPS	Lipopolysaccharide
NO	Nitric oxide
CON-A	Concanavalin A

Introduction

Euphorbia tirucalli L. (ET) is an ornamental plant of euphorbiaceae family, widely grown as an unarmed shrub in India and Africa. Plant latex is used in the folk medicine for the treatment of gastrointestinal disorder, epilepsy, sexual impotence, asthma, wounds, rheumatism, toothache, hemorrhoids, tumor and cancer (Gibriella et al. 2008; Wealth of India 1952; Mwine and Van 2011; Sauaia et al. 2013). ET latex extract depicted efficacy in murine tumor models (Valadares et al. 2006; Franco-Salla et al. 2016). It has shown to decrease the polymerization of tubulin to exhibit its anticancer activity (Khaleghian et al. 2010). Additionally, it has been found to exhibit pronounced antiviral activity (Betancur-Galvis et al. 2002). ET derived euphol (a tetracyclic triterpene) is shown to slow down gastric cancer cell growth inducing ERK1/2-mediated apoptosis (Lin et al. 2012). Moreover, it showed skin anti-inflammatory activity (Passos et al. 2013) and prevents colitis in experimental mouse models (Dutra et al. 2011). Previously, the biopolymeric fraction of ET has demonstrated in vivo anti-arthritic activity by down regulating production of IL-2 and IFN-γ (Bani et al. 2007). Also, this plant has been shown to modulate both the lymphocyte proliferation and myelopoiesis in in vitro cell culture assay (Llanes-Coronel et al. 2011; Valadares et al. 2006). In lieu of its reported cytotoxic activity, suppression of lymphocyte function and down regulation of inflammatory cytokines, it can be correlated with its traditional medicinal usage in various inflammatory disorders like asthma, tumors, wounds, rheumatism, toothache, and hemorrhoids. Interestingly, it has been reported that topical application of euphol, a tetracyclic triterpene isolated from the latex of E.T significantly inhibited experimental ear edema and skin inflammation by decreasing leukocyte influx, MIP-2 levels, protein kinase (ERK) activation and cyclooxygenase-2 (COX-2) activity (Passos et al. 2013).

Asthma is a chronic inflammatory disease of the airways (Opina and Moore 2017) caused by a very complex interaction between inflammatory cells and mediators, environmental pollutants, etc. It affects over 300 million individuals' worldwide, particularly aged people and children. It can be controlled temporarily by inhaled corticosteroids and β2 agonist bronchodilators. However, allopathic treatment that can cure it permanently is still far from reach (Opina and Moore 2017). Herbal therapy as alternative medicine has retrieved their popularity, efficacy and safety aspects to treat asthma as has been supported by controlled clinical studies (Huntley and Ernst 2000).

Symptomatically, pain may be manifested spontaneously or following a challenge with noxious or innocuous stimulation or after damage to, or alterations in sensory neurons (Woolf and Mannion 1999). Nonsteroidal anti-inflammatory drugs (NSAIDs) are the class of analgesic compounds most widely used to treat mild-to-moderate pain following injury, disease or minor surgery (Svensson and Yaksh 2002). Opioid agonists, carbamazepine, gabapentine, and serotonin re-uptake inhibitors have been used in case of severe pain management (Campbell and Meyer 2006). Currently, the medical practitioners are prescribing NSAID (Non steroidal anti-inflammatory drugs) and immunosuppressants to treat inflammatory and arthritic diseases, but these drugs cause serious side effects such as gastric mucosal damage, water and salt retention (phenylbutazone, oxyphenyl butazone) and even, possibly, carcinomas (Rostom et al. 2009). All these lacunae necessitate continuous search for new nociceptive pain killer and potential anti-inflammatory agents.

The literature supports that significant amount of phytoconstituents like steroids and terpenoids are present in ET root. These steroid and terpenoid components possess a glucocorticoid-like activity that may alleviate pain and inflammation in patients as earlier reports highlighted their role in lymphocyte proliferation (Llanes-Coronel et al. 2011) and wound healing (Santos et al. 2013). Some literature also reveals over immunosuppressant (Sugiura et al. 1994; Imai et al. 1994), which are evident in glucocorticoids therapeutics. Thus, we were encouraged to derive steroid and terpenoid rich fractions from the crude hydroalcoholic extract of the ET root to investigate its anti-inflammatory potentiality. We examined whether ET root derived steroid and terpenoid rich fractions are capable of healing pain, inflammation, arthritis and also asthmatic features in the established experimental models (Palit et al. 2016).

We determined the anti-inflammatory and analgesic effects of STF-HAETR and investigated the role of various inflammatory cytokines (TNF-α, IL-6, IL-12) involved therein. It has been postulated that the activation of phagocytes leads to the production of both reactive oxygen and nitrogen species which activates several proteases causing increased destruction of tissues in various inflammatory disorders (Chou 1997). We also assessed the COX inhibition activity and iNOS activity to elucidate the anti-inflammatory mechanism exhibited by ET. Since, suppression of inflammatory cytokines cause inhibition of migration of leucocytes leading to protection against the vascular permeability in acute inflammation related asthma and arthritis by reducing iNOS and COX expression (Ganju

et al. 2003; Hughes et al. 1999). To understand the pharmacodynamic role of STF-HAETR against arthritis, we also assessed the proteinase inhibition and protein denaturation study (Brown and Mackey 1968; Mizushima and Nakagawa 1966; Grant et al. 1970). Because protein denaturation and collagenase like proteinase induced cartilage tissue damage occur in the arthritis and associated joint inflammatory changes. The protective role of STF-HAETR against histamine induced bronchoconstriction in isolated tissue was further conducted to investigate the in vitro anti-asthmatic effect of this bioactive fraction. The cell membrane stabilizing activity was checked to elucidate the possible defensive role of STF-HAETR against inflammatory cascades associated with asthma and other etiological inflammatory disorders such as arthritis.

Materials and methods

Experimental animals

Albino mice of either sex weighing 20–25 g, healthy male albino adult rats weighing 150–230 g were used for in vivo pharmacological screenings. Animals were approved by Institute animal ethical committee (Ref. no. BCRCP/IAEC/6/2013) and kept under standard laboratory conditions for pharmacological evaluation. Carrageenan-induced acute-inflammation in albino rat model which was used to assess the anti-inflammatory activity of STF-HAETR. Hot plate model and acetic acid mediated peritoneal constrictions develop central and peripheral nociceptive pain in albino mice that appraise the analgesic activity of STF-HAETR as per established model.

Chemicals

All chemicals and reagents used in the present study are of analytical grade, purchased from SRL and SIGMA, E-Merck, India.

The root of *Euphorbia tirucalli* Linn along with whole plant was collected from the local area of Durgapur and prepared herbarium sample of the plant. The test plant was identified by the in-house Botanist through taxonomical identification procedure and preserved as voucher specimen reference as BCRCP/PBI/2/2013.

Extraction and preparation of steroid–terpenoid rich fraction from *Euphorbia tirucalli* root

Air-dried triturated root powder of *Euphorbia tirucalli*, 1200 g (W_i) was taken for hydro-alcoholic extraction through cold maceration process. The whole powder was taken in 5-L conical flask and 4 L of 70% ethanol solution was transferred into the flask. Entire soaking of dry powder into the extracting solvent, the menstruum was shaken continuously for 3 h and then kept at 4 °C room for 15 days. The solution was kept shaking with an agitator to disperse out the phytoconstituents into the solvent efficiently at every 3 days interval. After 15 days, the hydro-ethanol extract was shifted into another container. Remaining phytocompounds were dragged out from marc (residual cellular debris available after extraction from organized dried powdered stem) through re-maceration with fresh solvents. The whole solution was filtered. The filtrates were evaporated to dryness through rotary vacuum evaporator to obtain dry crude drug residue (W_f) containing phyto-extract. Finally, the % of yield of extractive value (W_e) was calculated as per indicated formula. $W_e = 100 \times W_f/W_i$. The extract was partitioned via *n*-hexane and ethyl-acetate solvents to afford two fractions of weight 75 and 50 g, respectively. *N*-hexane fraction was undertaken for silica gel column chromatography, eluted with hexane–acetone (95:5–5:95) to obtain ten fractions (A–J). Those fractions were assessed and the existence of steroid and terpenoid was confirmed by thin layer chromatography (TLC) and phytochemical test. Finally, the fractions were mixed together and evaporated to dryness in rotary vacuum to obtain dry residue of 13.6 g that coded as STF-HAETR and validated by phytochemical test and thin layer chromatography.

Phytochemical test and thin layer chromatographic study of STF-HAETR

Extracted crude phyto-extract was screened out to investigate the presence of phytochemical groups such as alkaloid, steroid, flavonoids, terpenoids, glycosides, saponin, and tannins on it in accordance with (Paech and Tracy 1955). TLC was undertaken in both crude extract and STF-HAETR extract to develop a chromatogram in mobile phase in chloroform: methanol (9:1) and chloroform: ethyl acetate: methanol (2:4:4) as per the method followed by Harborne 1998. R_f value was calculated from the developed chromatogram to characterize the extract. To identify the specific phytochemical group and locate their position in the developed chromatogram, different chemical spraying reagents were sprayed into the TLC plate. To identify the specific phytochemical group and locate their position in the developed chromatogram, two chemical spraying reagents like vanillin–phosphoric and *p*-anisaldehyde sulfuric acid were applied into the TLC plate. Existence of phytochemical groups was confirmed through changing of the band color at their corresponding R_f at post-treatment of spraying reagent. The chromatogram of STF-HAETR was compared in same solvent system with the standard phytomarkers of Lupeol and β-sitosterol.

Phytochemical characterization

HPTLC chromatogram was carried out for chemical characterization. Scanning wavelength 366 nm; mobile phase benzene: ethyl acetate (7:3) in formic acid 0.1%. The non-solidified STF-HAETR fraction was filtered, concentrated to 4 mL and used for HPTLC.

In vitro pro-inflammatory cytokine and nitric oxide evaluation

Peripheral blood mononuclear cells (PBMCs) were separated from venous blood of healthy volunteer by Histopaque-1077 (Sigma, Israel) gradient centrifugation. The cells were washed and cultured in RPMI-1640 medium (sigma) containing 1% penicillin, streptomycin, nystatin, and 10% fetal calf serum (FCS). PBMCs (2×10^6) were suspended in 1 mL of complete medium. For the induction of IL-12, TNF-α, and IL-6, the cultures were incubated for 24 h with 20 ng/mL of lipopolysaccharide (LPS; Lipopolysaccharides *W E. coli 055:B5*, Sigma) and CON (Concanavalin) A (2.5 µg/mL). At the end of LPS incubation, STF-HAETR extract was added at graded concentrations (10, 25, 50 and 100 µg/mL) for 24 h. At the end of the incubation period, the cells were removed by centrifugation at 1500g for 10 min; the supernatants were collected. IL-12, TNF-α and IL-6 were assayed for cytokine content as per manufacturers protocol through standard calibration curve of corresponding cytokines (BD pharmingen) by ELISA method and nitric oxide content were estimated with Griess reagent (Ding et al. 1988).

Assessment of pro-inflammatory mediators' iNOS (inducible nitric oxide synthase) by western blot analysis

The experiments were carried-out using mouse macrophage cell lines RAW 264.7 cultured in complete RPMI medium with the 100 U/mL penicillin, 100 mg/mL streptomycin and 10% heat-inactivated fetal calf serum. After stimulation with LPS (1 µg/mL) + IFN-γ (30 ng/mL) and pre-treatment with STF-HAETR (34 µg/mL; IC$_{50}$) for 2 h, cells were rinsed three times in PBS before extraction. Whole cell lysate (50 µg protein/lane) was electrophoresed through an 8% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis and electro blotted onto PVDF (polyvinylidene fluoride) membranes. Membranes were blocked with 5% BSA/TBST (Bovine serum albumin/Tris-Buffered Saline and Tween 20) and probed with a polyclonal anti-mouse iNOS antibody (1:1000 dilution) followed by goat anti-mouse IgG conjugated to horseradish peroxidase (1:7000 dilution, Santa Cruz Biotechnology) in TBST. After extensive washing, ECL detection was performed according to the manufacturer's instructions (Thermo Scientific). The same membrane was stripped using stripping buffer (Thermo scientific) and probed for β-actin as a loading control.

In-vitro cyclooxygenase (COX) inhibition appraisal of STF-HAETR

Assay of COX-1 and COX-2 inhibition activity was undertaken using a commercially available colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical Company, Ann Arbor, MI; lot 184104) containing the recombinant enzyme. STF-HAETR was added to the reaction system at graded concentrations (ranging from 10, 20, 50 and 100 µg/mL), dissolved in 0.2% DMSO, and prepared just before use. COX activity was evaluated using N, N, N', N'-tetraethyl-p-phenylenediamine (TMPD) as a co-substrate with AA (reduction of PGG2 to PGH2). TMPD oxidation was observed spectrophotometrically with a 96-well plate reader at 590 nm. No colorimetric change was monitored in control incubations that were performed by omitting enzymes or with heat-denatured enzymes and without fraction in combination with TMPD. IC$_{50}$ value of STF-HAETR against the COX-1 and COX-2 activities was evaluated by the linear regression analysis from the % of COX inhibition vs. concentration curve.

In vitro anti-asthmatic evaluation

Freshly isolated adult goat trachea was obtained from the local slaughter house and preparations were made for evaluation (Nag Chaudhari and Lahiri 1974). The tracheal chain was suspended in bath of Kreb's solution containing Sodium chloride 6.9, potassium chloride 0.35, Calcium chloride 0.28, Magnesium sulfate 0.28, sodium bi carbonate 2.1, Potassium Di hydrogen phosphate 0.16, and Glucose 2.0 gL-1, which was continuously aerated and maintained at 37 °C. The whole preparations were adopted for assay as per the method (Dhonde et al. 2008). A dose–response curve for histamine was taken in variant molar concentrations, by maintaining a 5 min time cycle. After obtaining a dose–response curve of histamine (2.5 µg/mL) from 0.1 to 1.6 mL of Kreb's solution in the organ bath of trachea, 100 µg/mL of STF-HAETR were added to the respective reservoir with a sub maximal dose of histamine. Percent of maximum contractile responses were plotted to record the dose response curve of histamine, in absence and presence of STF-HAETR.

In vitro inhibition of protein denaturation, proteinase inhibitory action and membrane stabilization studies

Protein denaturation assay was conducted to evaluate the anti-arthritic potential in vitro system. The final reaction mixture (5 mL) consisted of 2.40 mL solution of 5% aqueous bovine serum albumin and 0.10 mL test sample solution containing STF-HAETR (10, 25, 50 and 100 μg/mL of final volume) dissolving in 0.2% DMSO (respect to final reaction volume). 0.10 mL blank 0.2% DMSO was used in untreated control group instead of test fraction. pH was adjusted at 6.3 using a small amount of 1 N HCl. All the samples were incubated at 37 °C for 20 min and then heated at 57 °C for 30 min. After cooling the samples, 2.5 mL phosphate buffer saline (pH 6.3) was added to each tube to make up the final reaction volume (5 mL). Turbidity was measured including positive controls containing aspirin (100 μg/mL) spectrophotometrically (Sadique et al. 1989) at 660 nm along with the untreated controls. While product control group lacked bovine serum albumin as per method adopted (Sadique et al. 1989). The percentage inhibition of protein denaturation was calculated as follows:

$$\text{Percentage of inhibition} = 100 - (\text{O.D. of test} - \text{O.D. of product control}) \times 100/\text{O.D. of untreated control.}$$

Trypsin mediated casein degradation experiment was conducted to evaluate the proteinase inhibitory potential of the test sample (STF-HAETR). This assay was performed to simulate the in vitro anti-inflammatory and anti-arthritic activity of STF-HAETR. To design the assay, 0.06 mg trypsin was dissolved in 1.0 mL 25 mM tris–HCl buffer (pH 7.4). 10, 25, 50 and 100 μg/mL (respect to final reaction volume (3 mL)) of test sample solution of STF-HAETR was prepared dissolving in 0.4% V/V DMSO in 1 mL of aqueous solution and added with the 1 ml of trypsin solution in 25 mM tris–HCl buffer (pH 7.4). The reaction mixture of 2 mL was incubated at 37 °C for 5 min, and then 1.0 mL of 0.8% (w/v) casein was added. The final reaction mixture was further incubated for 20 min at 37 °C. 1 mL blank solution of 0.4% V/V DMSO was used as untreated control group instead of test fraction 2.0 mL of 70% (v/v) perchloric acid was added to the each reaction mixture tube to terminate the enzymatic reaction. The cloudy suspension was centrifuged. The absorbance of the supernatant of each group of the solution was measured at 280 nm against buffer as blank (Oyedapo and Famurewa 1995). The percentage proteinase inhibition was calculated as per the following formula and compared with positive controls of aspirin (100 μg/mL).

$$\% \text{ of inhibition} = 100 - [(\text{O.D of test solution} - \text{O.D. of only STF} - \text{HAETR fraction without casein} - \text{trypsin enzyme reaction}) \div (\text{O.D. of untreated control}) \times 100].$$

RBC membrane stability assay was carried out to simulate the in vitro anti-arthritic assessment. The principle was involved through the stabilization of human red blood cell (RBC) membrane by hypotonic solution induced membrane lysis. The assay mixture (4.5 mL) was consisted of 2 mL hypotonic saline (0.25% NaCl), 1 mL 0.15 M phosphate buffer saline (pH 7.4) and 1.0 mL of test sample solution of STF-HAETR [10, 25, 50 and 100 μg/mL of final volume (4.5 mL)] dissolving in 0.2% DMSO v/v solution in aqueous base. 0.5 mL HRBC suspension (10% v/v) in normal saline was added. For untreated control group, 1 mL of 0.2% DMSO v/v solution in aqueous base was used instead of test fraction solution while product control group lacked red blood cells suspension. The final reaction mixture volume was incubated at 56 °C for 30 min. All the tubes were cooled down under running tap water for 20 min. The mixtures were then centrifuged and the absorbance of the supernatants was observed at 560 nm (Brown and Mackey 1968).

Percent membrane stabilizing activity was calculated as follows:

$$\text{Percent stabilization} = 100 - (\text{O.D. of test fraction} - \text{O.D. of product control})/\text{O.D. of untreated control} \times 100$$

The untreated control represents 100% lysis by inducing 2 ml of hypotonic solution of 0.25% NaCl. The result was compared with the positive control of aspirin (100 μg/mL).

In vivo assessment of anti-nociceptive pain of STF-HAETR

Twenty-four hours prior to actual testing, 50 mice (20–25 g) received 0.6% glacial acetic acid intraperitoneally by 10 mL/kg dose basis. Animals were scrutinized for writhing movements to evaluate peripheral anti-nociceptive pain effect (Collier et al. 1968).

Actually, 0.6% acetic acid was used to induce abdominal constrictions, as referred to as peripheral nociceptive pain, in the experimental mouse. Only those showing one or other type of writhing movements (positive responders) were chosen for the test on the next day. On the test day the positive responders received STF-HAETR at a dose of 30 and 60 mg/kg non-lethal oral dose (Selected as 1/10th of estimated LD_{50} dose, i.e., 600 mg/kg in mice as per pharmacology rule; http://www.samipharmapk.com). The test drug was dissolved in liquid solution of 0.2(M) phosphate buffered saline containing 0.2% tween 80 and 0.5% sodium

CMC (carboxymethyl cellulose) and administered single dose for one time half an hour prior to glacial acetic acid challenge intraperitoneally. Indomethacin was used as positive control at 5 mg/kg and administered by oral route for one time as single dose. Indomethacin was dissolved in 0.2 (M) phosphate buffered saline containing 0.2% tween 80 and 0.5% sodium CMC. Each mouse was then observed for the total number of stretching episodes or writhe for 15 min following glacial acetic acid injection. The mean value of total number of writhes due to abdominal constrictions for each mouse of all experimental groups (6 mice per group) was reported. The percentage of protection for two different doses was evaluated with respect to untreated controls (receiving glacial acetic acid only without additional drug). The number of writhes in each treated group was compared with untreated control group. The number of writhes was recorded and the percentage of protection was calculated using the following formula.

% protection = (Mean writhes in control untreated group
  − mean writhes in treated group/mean writhes in control)
  × 100.

Central anti-nociceptive pain assessment was carried out by the hot-plate technique (Turner 1965) using swiss albino mice (25–35 g) of either sex selected by random sampling in hot-plate apparatus (53 ± 0.5 °C). The reaction time was counted as the time interval between placing the animal on the hot plate and when it licked its hind paw or jumped from hot plate. Aceclofenac, at a dose level of 20 mg/kg, was administered intraperitoneally for one time as a positive control, standard analgesic drug for comparison and validation of the model. STF-HAETR was given at 30 and 60 mg/kg, orally as single dose for one time. The reaction time was recorded in second after placing the tested animal in Eddy's hot plate; it takes how much time to lick its paw for the first time at post treatment. The central analgesic effect was expressed as a percentage of protection in the test group compared to untreated control groups, by counting of the number of paw licking at each experimental group. The untreated control group was fed with the only vehicle containing liquid solution of 0.2(M) phosphate buffered saline including 0.2% tween 80 and 0.5% sodium CMC without STF-HAETR.

In vivo anti-inflammatory evaluation

The method was adopted as described (Winter et al. 1962) with slight modifications of carrageenan-induced rat hind paw edema model. The albino rats weighing between 150 and 180 g was starved for 18 h prior to the experiment. The animals were weighed, marked for identification and divided into four groups, containing six animals at each group. Edema was induced in the left hind paw of all rats by subcutaneous injection of 0.1 mL of 1% (w/v) carrageenan in distilled water into their footpads. The 1st group was kept as control and was given the respective volume of the vehicle (0.2 M PBS). The 2nd and 3rd groups were received STF-HAETR at a dose of 50 and 100 mg/kg (Chosen as 1/10th of estimated LD_{50} dose, i.e., 1000 mg/kg in mice as per pharmacology rule; http://www.samipharmapk.com) 1 h before carrageenan injection. The last group (standard) was received indomethacin in a dose of 10 mg/kg intraperitoneally (Mino et al. 2004). The paw volume of each rat was measured immediately by mercury plethysmometer, before carrageenan injection and 1, 2 and 3 h post-administration of carrageenan. The edema rate and inhibition rate of each group were estimated as follows: edema rate $(E)\% = V_t − V_0/V_0 × 100$, inhibition rate $(I)\% = E_c$ $E_t/E_c × 100$, where V_0 is the volume before carrageenan injection (mL), V_t is the volume at t hours after carrageenan injection (mL), E_c, E_t are the edema rate of control group and treated group, respectively.

Acute toxicity study and liver, kidney and heart marker enzyme analysis

30 rats were randomly divided into five groups of six rats per group. Graded doses of STF-HAETR (100–2000 mg/kg) were allotted for four groups. Group 1 obtained liquid solution of 0.2(M) phosphate buffered saline containing 0.2% tween 80 and 0.5% sodium CMC (carboxymethyl cellulose) intraperitoneally. Groups 2–5 were treated with total STF-HAETR (100, 500, 1000 and 2000 mg/kg, respectively) intraperitoneally. Similarly, the toxicity study was carried out with 30 mice by dividing them into 5 groups consisting of six mice per group. The test mice of 4 groups were received intraperitoneal dose of 60, 120, 400, and 800 mg/kg. Group 1 was treated with liquid solution of 0.2(M) phosphate buffered saline containing 0.2% tween 80 and 0.5% sodium CMC (carboxymethyl cellulose). All animals were observed for a mortality rate over a 2-month period. The serum enzymes like urea, creatinine, SGPT, SGOT, ALT and blood LDH were assessed after completing observation at 2 months post treatment.

Statistical analysis

The IC_{50} was calculated from nonlinear regression analysis using GraphPad Prism 5.0 software. In other cases, unpaired student t test was applied to estimate the differences among the treatment groups and untreated controls. P values < 0.05 were considered to be statistically significant. All the results were expressed as mean ± standard error (SEM).

Results

Chemical characterization of HAETR extract and STF-HAETR fraction by TLC and HPTLC investigation

Extraction through cold maceration procedure of *Euphorbia tirucalli root* in hydro-alcoholic solvent system (30:70) yielded 12.5%. Phytochemical screening of HAETR root indicated that the extract was enriched in steroids, terpenoids and tannins along with mild to moderate amount of alkaloids, saponins and glycosides. TLC study illustrated 6 bands in the HAETR extract in the chloroform:methanol (9:1) and chloroform:ethyl acetate:methanol (2:4:4) mobile phase with R_f of 0.15, 0.23, 0.67, 0.87, 0.90, 0.97 and 0.16, 0.28, 0.70, 0.90, 0.93, 0.98, respectively. Among them, R_f of 0.67 and 0.87 confirmed the presence of steroid and terpenoid, respectively, as evidenced by spraying with vanillin–phosphoric acid and *p*-anisaldehyde spraying reagent in the chloroform: methanol (9:1) mobile phase (Table 1).

Phytochemical screening suggested that our processed STF-HAETR fraction contained only steroids and terpenoids and with apparently no other secondary metabolites. TLC chromatogram suggested that the separated fraction of STF-HAETR consisted of 4 bands with R_f value of 0.72, 0.78 (steroid) and 0.91, 0.98 (terpenoid) as presented in Table 2. Lupeol and beta-sitosterol as a phytomarkers were matched with the R_f values obtained from the bioactive fractions of STF-HAETR as 0.78 and 0.91 (Table 2).

As illustrated in Fig. 1, preliminary HPTLC study indicated 11 distinct peaks in the chromatogram. Out of these, the seven major peaks with R_f: of 0.03, 0.12, 0.39–0.46 and 0.62–0.74 was found. It also showed 4 minor peaks at R_f of 0.21, 0.30, 0.52 and 0.56, respectively. When we assessed all these peaks in spectral scan (graphical) we found that as the repeat increased the peak become distinct and sharper suggesting that the specific compound in the drug becomes more refined/purified. Results are shown in Fig. 1a, b.

Assessment of in vivo nociceptive assay

The results illustrated in Fig. 2 shows that STF-HAETR executed a dose-dependent inhibition of the acetic acid-induced abdominal constrictions in mice, demonstrating $35.72 \pm 9.5\%$ protection at 30 mg/kg. Maximal inhibition of $69.37 \pm 2.5\%$ abdominal constrictions was displayed by 60 mg/kg of STF-HAETR. Whereas the intraperitoneal treatment of mice with positive control, indomethacin at 5 mg/kg, exhibited $70 \pm 1.1\%$ inhibition of writhing compared to untreated controls. The results indicated that maximal protection of abdominal constrictions by STF-HAETR was almost similar to marketed standard drug (Indomethacin). Furthermore, STF-HAETR significantly increased the latency of paw licking time in Eddy's hot plate model to demonstrate the central analgesic activity in comparison to vehicle controls at 30 and 60 mg/kg body weight. Maximum protection was monitored by showing 95 s elapsing to the first pain response at 60 mg/kg doses of STF-HAETR, that were comparable to the reaction time (92 s.) observed with aceclofenac sodium (20 mg/kg) as represented in Fig. 3. Results pointed out that test bioactive fraction showed better protection against central nociceptive pain than aceclofenac sodium, a standard analgesic.

Anti-inflammatory evaluation

In carrageenan-induced animal models, STF-HAETR extract, reduced edema formation in third hour by 62.37% ($P < 0.01$) (50 mg/ka), %, and 84.42% ($P < 0.001$) (100 mg/kg) in a dose-dependent manner. This result also extended and radically improved up to the fifth hour

Table 1 Phytochemical screening and R_f evaluation of HAETR extract by TLC

Phytochemical compound		Hydro-alcoholic root extracts of ET					
		Chloroform: methanol (9:1)			Chloroform: ethyl acetate:methanol (2:4:4)		
		R_f	Spraying reagent	Color after spraying	R_f	Spraying reagent	Color after spraying
Alkaloid	+	0.15			0.16		
Tannin	+++	0.23			0.28		
Flavonoid	−						
Steroid	+++	0.67	Vanillin—phosphoric acid	Pink	0.70	Vanillin—phosphoric acid	Pink
Terpenoid	+++	0.87	*p*-Anisaldehyde—sulfuric acid	Red	0.90	*p*-Anisaldehyde—sulfuric acid	Red
Saponin	++	0.90			0.93		
Glycoside	++	0.95			0.98		
Amino acid	−						

Table 2 Phytochemical screening and R_f evaluation of STF-HAETR fraction by TLC

Phytochemical compound	STF-HAETR fraction chloroform:methanol (9:1)		
	R_f	Spraying reagent	Color
Alkaloid	−		
Tannin	−		
Flavonoid	−		
Steroid	+++	0.72, 0.78	Vanillin—phosphoric acid	Pink
Terpenoid	+++	0.91, 0.98	p-Anisaldehyde—sulfuric acid	Red
Saponin	−		
Glycoside	−		
Amino acid	−		
β-Sitosterol	+++	0.78	Vanillin—phosphoric acid	Pink
Lupeol	+++	0.91	p-Anisaldehyde—sulfuric acid	Red
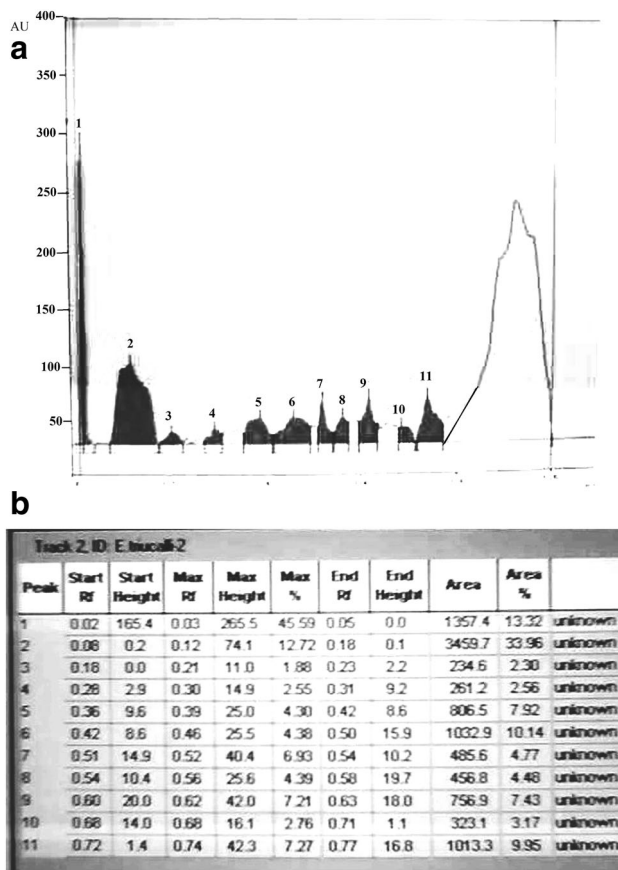
Fig. 1 Phytochemical characterization of STF-HAETR by HPTLC chromatogram

($P < 0.001$) exhibiting 72.56 and 96.97% protection towards inflammatory paw edema, respectively. Most interesting finding is that our STF-HAETR extract
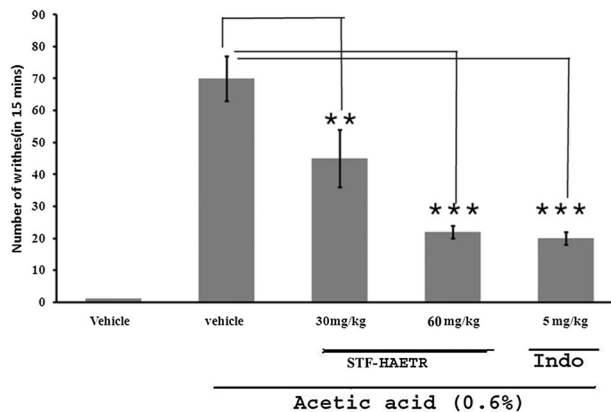
Fig. 2 Peripheral analgesic activity of STF-HAETR was assessed on 0.6% acetic acid induced writhing in mice model compared to standard drug indomethacin. The vehicle was used as 0.2 (M) phosphate buffered saline containing 0.2% tween 80 and 0.5% sodium carboxy methyl cellulose for dissolving the test sample (STF-HAETR) and indomethacin. The results were expressed as mean ± SEM ($n = 6$ mice per group), representative of three similar experiments. ***$P < 0.0001$, **$P < 0.001$, considered as significant difference compared to untreated vehicle controls, assessed by two-tailed unpaired student t test

displayed improved and higher anti-inflammatory activity in comparison to reference drug indomethacin as shown in Fig. 4.

Effect of plant fraction on LPS-induced NO and pro-inflammatory cytokine production

Nitric oxide formation by 10 ng/mL LPS-stimulated PBMC cells was found to be significantly inhibited ($P < 0.05$) by STF-HAETR in a dose-dependent manner with 25, 32.5, 70.84 and 80% reduction at 10, 25, 50 and 100 μg/mL, respectively, compared to untreated controls (Fig. 5) which confirms its anti-inflammatory activity. As shown in the Fig. 5 cells treated with STF-HAETR led to 8, 21, 68.5, and 76.32% reduction in nitrite production, respectively, compared to positive control, indomethacin, a standard NO inhibitor.

Human PBMC derived macrophage cells treated with LPS and CON A showed a substantial up regulation in the levels of IL-12, TNF-α and IL-6 in the culture supernatants (Fig. 6). STF-HAETR was capable to diminish the levels of all three cytokine levels significantly in a dose-dependent manner. It demonstrated highest level, i.e., 44 (97.73%) fold reduction ($P < 0.0001$) on LPS + CON-A induced IL-6 production at 100 μg/mL compared to untreated controls. Other doses like 10, 25, 50 μg/mL suppressed 2.26 ($P < 0.001$), 7.33 ($P < 0.0001$) and 22 ($P < 0.0001$) fold of IL-6 production in comparison to untreated controls, respectively (Fig. 6a). Vis a vis STF-HAETR also reduced TNF-α release by 44.45%

Fig. 3 The central analgesic activity of STF-HAETR is represented in terms of reaction time, i.e., recorded in seconds after placing the tested animal in Eddy's hot plate. Time to lick its paw for the first time following STF-HAETR post treatment at 30 and 60 mg/kg are reported. The results are expressed as mean $\pm$ SEM. ($n = 6$ mice per group), representative of three similar experiments. Aceclofenac, a positive control at 20 mg/kg dose, was given intraperitoneally on experimental mouse to validate the ex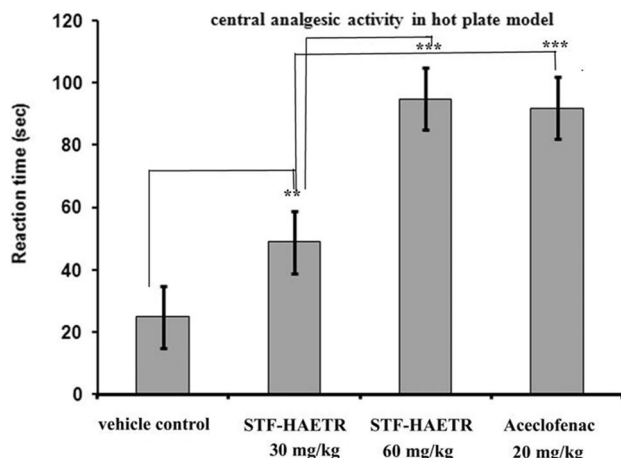perimental model of central anti-nociceptive assay and to monitor whether the experiment are proceeding in the right way or not followed by the treatment of STF-HAETR for concluding the results. Aceclofenac has been undertaken to check whether hot plate generated heat induces central nociceptic pain or not and subsequently the ameliorative results shown by the STF-HAETR can be concluded statistically comparing with the standard drug, aceclofenac results
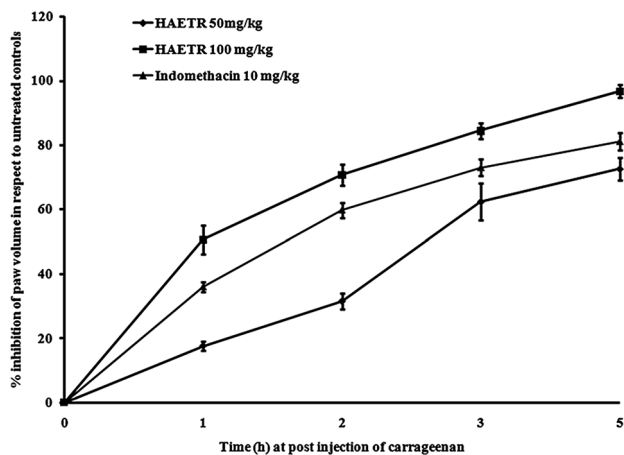
Fig. 4 Alleviating effect of STF-HAETR on carrageenan-induced rat paws edema for assessing the acute anti-inflammatory effect. The results are expressed as mean $\pm$ SEM ($n = 6$), representative of two similar experiments. The observed results of % inhibition of paw edema volume are presented at 1, 2, 3, 4 and 5 h at post treatment duration

($P < 0.001$) and 11.12% (Fig. 6b) at 25 and 10 μg/mL. Similarly, down regulation of the well known pro-inflammatory cytokine TNF-α by 72.23% ($P < 0.0001$), 61.12% ($P < 0.0001$) was observed on exposure to 100 and 50 μg/mL of STF-HAETR extract (Fig. 6b). STF-HAETR
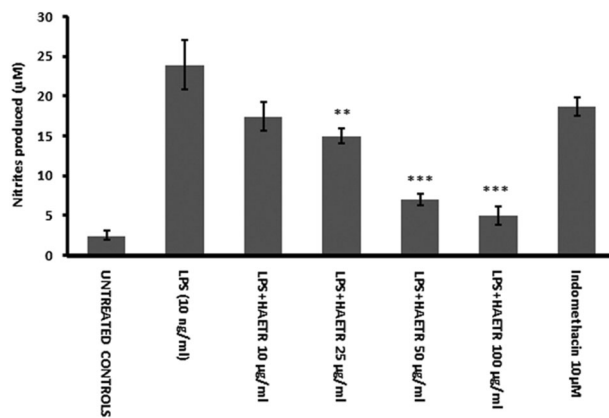
Fig. 5 LPS stimulated NO production from PBMC derived cultured macrophages was repressed by STF-HAETR in a dose-dependent manner. The results are expressed as mean $\pm$ S.E.M. ($n = 3$), representative of three similar experiments. ***$P < 0.0001$, **$P < 0.001$, considered as significant difference when compared with LPS-treated controls without STF-HAETR, evaluated by two tailed unpaired student t test

fraction also significantly inhibited IL-12 release by 7.15%, 28.58%, 53.57 ($P < 0.001$) and 64.29% ($P < 0.0001$) at 10, 25, 50 and 100 μg/mL, respectively (Fig. 6c).

STF-HAETR decreased the expression levels of pro-inflammatory mediator, iNOS

We investigated the effects of the STF-HAETR on iNOS expression by Western blot. Upon LPS (1 μg/mL) and IFN-γ (30 ng/mL) treatment there was enhanced expression of iNOS (Fig. 7, lane 2). Interestingly, STF-HAETR down-regulated LPS + IFN-γ-induced iNOS expression at IC_{50} dose compared to the untreated controls (Fig. 7, lane 3). This study confirmed the anti-inflammatory effect of STF-HAETR that down-regulates inducible nitric oxide synthase and hence nitric oxide radical generation.

Effects of STF-HAETR extract on COX activity

The inhibitory effects of STF-HAETR on COX-mediated TMPD oxidation activity were evaluated using purified COX as enzyme sources. STF-HAETR did not inhibit significantly the COX-1 activity at 100 μg/mL. COX-2 activity was strongly inhibited by STF-HAETR treatment demonstrating IC_{50} of 25.14 μg/mL. The inhibition of COX-2 by the STF-HAETR was markedly higher than that of COX-1. Therefore, STF-HAETR itself is recommended to be a selective COX-2 inhibitor with selectivity index of 6.55. This result was quit comparable to celecoxib, a selective COX-2 inhibitor as presented in Table 3. Dose–response curve of the test fraction (STF-HAETR) for COX-1 and COX-2 inhibitory activity had been presented in Fig. 8.

Fig. 6 Impact of STF-HAETR on the LPS + CON-A induced release of pro-inflammatory cytokines IL-6 (**a**), TNF-α (**b**) and IL-12 (**c**) from PBMC derived cultured macrophages at dose-dependent manner. The cytokine levels were estimated by ELISA method. The results are expressed as mean ± SEM ($n = 3$ per group), representative of two similar experiments. ***$P < 0.0001$, **$P < 0.001$, considered as significant difference when compared to LPS + CON-A treated controls without STF-HAETR, assessed by two tailed unpaired student t test
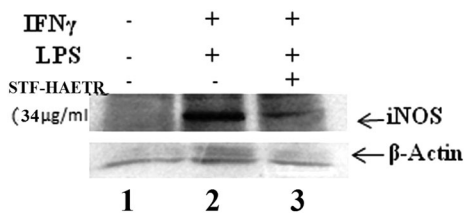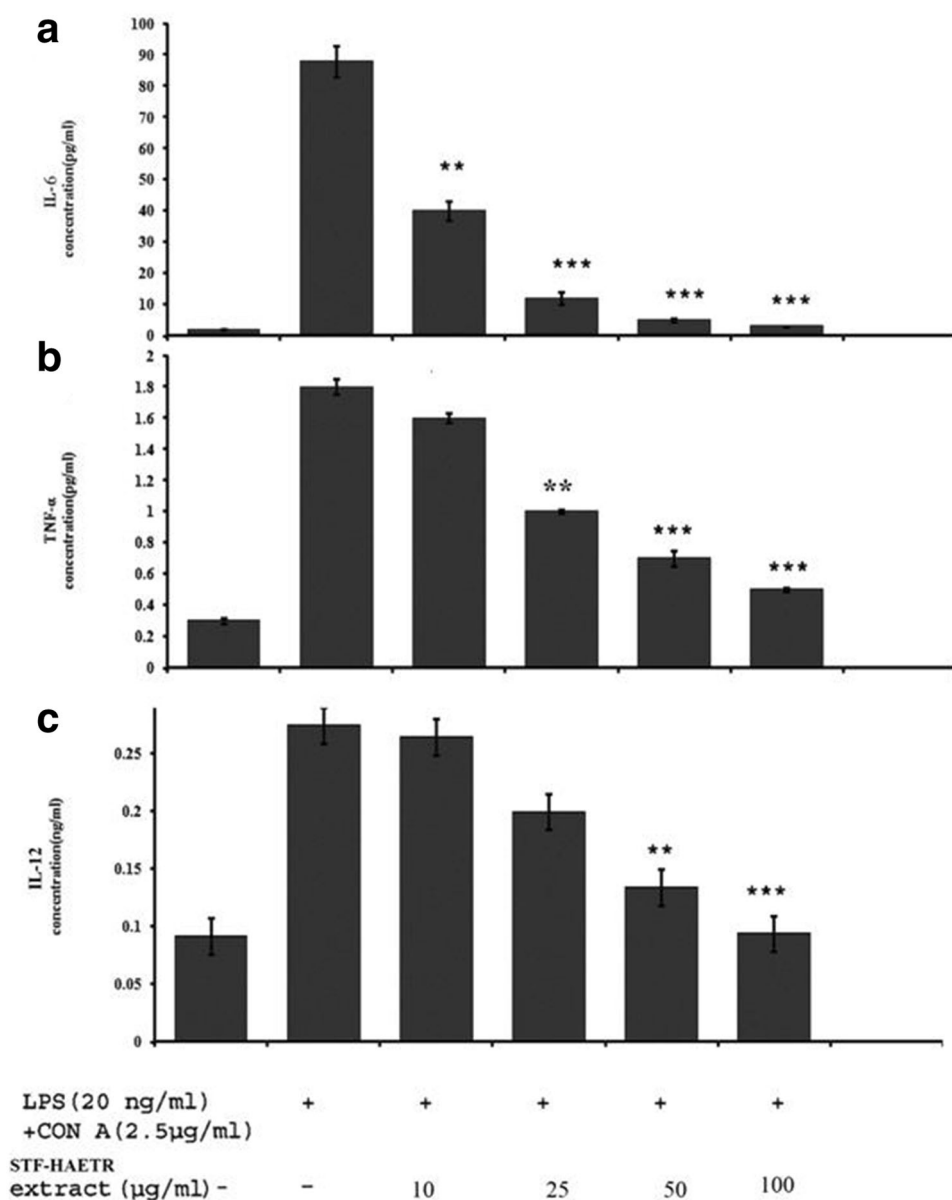
Fig. 7 Activity of STF-HAETR on LPS + IFN-γ induced iNOS protein expression in RAW264.7 cells by western blot technique. RAW 264.7 cells were stimulated by LPS (1 μg/mL) and IFN-γ (30 ng/mL) prior to treatment with STF-HAETR (34 μg/mL). After 24 h post treatment, immunoblots were run using antibody against iNOS using β-actin as loading control. STF-HAETR had significantly decreased the iNOS protein expression (lane 3) compared to LPS + IFN-γ stimulated controls without STF-HAETR (lane 2)

In vitro anti-asthmatic assessment of STF-HAETR in histamine induced goat tracheal contraction

In vitro studies illustrated that STF-HAETR reduced the bronchoconstriction produced by histamine in the isolated goat tracheal tissue preparation. Dose response curve was plotted in absence and in presence of STF-HAETR. It was found that STF-HAETR inhibited the 0.2 mL histamine solution induced contraction with maximum percentage inhibition (51.37%) as presented in Table 4.

Effect of STF-HAETR on protein denaturation, proteinase inhibitory action and membrane stabilization

In vitro protein denaturation in BSA model was remarkably inhibited by STF-HAETR showing 12.56–75.67% protection at the doses ranging from 25 to 100 μg/mL, respectively. These findings were almost comparable to the results obtained by the reference drug aspirin at 100 μg/mL. Furthermore, STF-HAETR stabilized the hypotonic saline induced human RBC membrane lysis by 77.51% at 100 μg/mL, which was found to be 1.2 times more potent than the standard drug aspirin at equivalent dose (Table 5). STF-HAETR at 4 different graded doses (25, 50, 75 and 100 μg/mL) displayed significant protection against proteinase activity by 32.23, 46.35, 54.79 and 67.47% inhibition, respectively. Anti-proteinase activity of STF-HAETR was 1.67 times higher than the standard drug (aspirin) at similar dose of 100 μg/mL.

Discussion

Inflammatory disorders like arthritis and asthma are the diseases very much related to each other in respect of etiological roles of the pro-inflammatory cytokines in disease progression, which are also the overall mediators of pain and inflammation (Tesmer et al. 2008).

Since the biopolymeric fractions of the crude latex of *E. tirucalli* (Bani et al. 2007) has been reported to posses significant antiarthritic activity, so we wanted to explore the bioactive fractions derived from roots for the management of pain and inflammation cascade as organized drug, i.e., having the crude drug material which represent plant parts. In this present study, the total steroid and terpenoid rich fractions obtained from hydro-alcoholic root extract (HAETR) demonstrated a significant analgesic and anti-inflammatory effect in subsequent paw licking paw
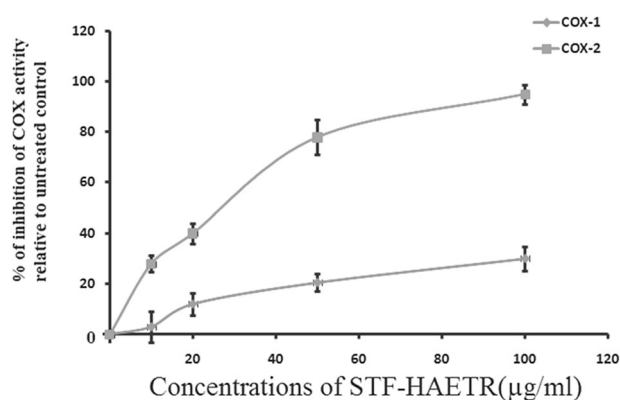
Fig. 8 the four different doses (ranging from 10 to 100 μg/ml) have been tested against bovine COX-1 and 2. The results are expressed as mean $\pm$ SEM ($n = 3$ per group), representative of two similar experiments. The IC_{50} values have been evaluated by linear regression analysis from the presented dose–response curve

edema and writhing models in mice compared to the standard drug as positive control. But specifically STF-HAETR had a more potent anti-inflammatory effect than analgesic effect, as a dose of 100 mg/kg showed a significant (96.97%) suppression of acute paw edema, while with modest analgesic actions at similar dose in paw licking and writhing responses. Since, hot plate experimental method induced pain is much more specific for the drugs which acts centrally and is one of the models normally used for studying central nociceptive assay (Vale et al. 2004). But carrageenan, an inflammatory agent is basically used as a pharmacological tool for evaluating hyperalgesia due to inflammation in rodents. Speaking of the anti-inflammatory action of glucocorticoids, which is very much evident in carrageenan-induced paw edema models (Enomoto et al. 2007) with modest effects in other models of analgesia, probably indicates the anti-inflammatory activity through an immunosuppressive effect and down regulation of the genes for pro inflammatory cytokine (Barnes 1998) COX-2 (Masferrer et al. 1990; Lee et al. 1992) and NO (Xin et al. 2011). STF-HAETR's potent anti-inflammatory activity

Table 3 Comparison of ICso values (ug/mL) of various COX inhibitors

Inhibitors	IC50		COX-l/COX-2 Ratio[a]	Reference
	COX-1	COX-2		
STF-HAETR	164.79	25.14	*6.55 (P < 0.001)*	This work
Celecoxib	10.15	0.17	*60.0 (P < 0.001)*	Tsai et al. (2006)
Diclofenac	0.02	0.1	0.31	Johnson et al. (1995)
Indomethacin	0.001	0.121	0.01	Tsai et al. (2006)

The ICso values for each of the inhibitors are taken from the published data carried out using ovine COX-1 and COX-2 as enzyme sources (see "Materials and methods")

[a] Proportion of the IC_{50} values for COX-1 and COX-2 can be used as an indication of the COX-2 selectivity of inhibitors. A COX-l/COX-2 ratio of more than 1 signifies preferential COX-2 selectivity. $P < 0.001$ is significant difference between COX-1 and COX-2, assessed by un-paired student t test

Table 4 Effect of STF-HAETR on histamine induced contraction on the isolated goat tracheal chain preparation was presented

Dose of Histamine (2.5 µg/mL) (mL µg/mL)	Isolated goat tracheal chain preparation	
	Control group % maximum contraction	STF-HAETR (100 % maximum contraction
0.1	22.89 ± 1.20	11.44 ± 0.70*
0.2	52.567 ± 4.567	25.56 ± 2.345*
0.4	73.56 ± 5.345	45.678 ± 3.345*
0.8	90.3456 ± 1.234	56.543 ± 3.254*
1.6	93.4321 ± 2.4567	63.567 ± 2.567*

All values are expressed as mean SEM of a sample size $n = 5$

*$P < 0.05$ was considered as level of significant compared to untreated controls

with analgesia is probably due to its specificity in suppression of inflammatory cytokines, COX-2 activity, with a modest action over supraspinal pain mediators as our in vitro studies showed. COX-2 especially plays a more prominent role than COX-1 in inflammation of adjuvant arthritis, and COX-2 derived PGs itself up regulates COX-2 and proinflammatory cytokines expression at site of inflammation (Anderson et al. 1996). Also in synovial tissues of patients with rheumatoid arthritis (RA) it has been reported that there is an increased expression level of COX-2, which is mainly modulated by the levels of proinflammatory cytokines like TNF-α (Crofford 1997). Moreover, STF-HAETR showed a potent inhibition of COX-2 enzyme, with no significant inhibition of COX-1. This may further justify STF-HAETR's anti-inflammation selectivity with lesser gastrointestinal and homeostatic side effects which are attributed to COX-1 inhibition (Mardini and Fitzerald 2001).

Previous studies showed the significant increase in the levels of COX-2 and TNF-α in paw edematous rat's tissues after carrageenan-induced inflammation compared to control rats (Lucetti et al. 2010; Nantel et al. 1999). Increased levels of NO and COX-2 expression are also reported to be associated with the second phase of paw edema in rats (Posadas et al. 2004). Our in vivo paw edema inflammatory

model reinforces, carrageenan induced local TNF-α, COX-2, NO and cytokines production in the affected paws. In this paw edema model of inflammation COX-2–derived PGs also appear to mediate a variety of pro-inflammatory effects including enhancement of cellular exudation, up regulation of pro-inflammatory cytokines and COX-2, etc. (Williams and Shacter 1997). Previous research also showed that cytokine especially IFN-γ synergistically with TNF-α stimulates COX-2m-RNA expression during macrophage activation in situation of inflammation (Arias-Negrete et al. 1995). Thus, the overall literature survey indicates that a dual vice versa synergistic stimulating effects between each other may occur among COX-2 derived prostaglandins and cytokines TNF-α and IFN-γ in inflammatory response.

In vitro selective COX-2 inhibitory effects compared to COX-1, resembles the in vivo anti-inflammatory action of STF-HAETR wherein it suppresses paw edema, may be by in vivo inhibition of COX-2 and TNF-α at the site of inflammation, as in vitro human PBMC cytokine results also depicts. To delineate further possible mechanism of anti-inflammatory effect of STF-HAETR, a broad spectrum inflammatory mediators like pro-inflammatory cytokines, nitric oxide, protease, etc., were assessed and validated in in vitro model, too.

Several researches also established that IL-12 synergizes with TNF-α and together induces the production of IFN-γ and pro-inflammatory cytokines. Moreover, IL-12 in the synovial fluid of patients of arthritis, positively correlated with TNF-α and IL-6 levels, thus IL-12 production is closely associated with the levels of pro-inflammatory cytokines in cases of chronic inflammation In our findings, alterations in the mediators of inflammatory cascade in human PBMC culture, showed primarily a significant suppression in the inflammatory cytokines TNF-α and IFN-γ, with a more potent suppression of IL-12. This in vitro results can be correlated to the in vivo anti-inflammatory effects obtained in our carrageenan-induced paw inflammation. Out of the three pro-inflammatory cytokines analyzed, STF-HAETR most potently diminished the levels of IL-12 than TNF-α and IFN-γ. As researches has

Table 5 Effect of STF-HAETR on inhibition of protein denaturation, proteinase inhibitory action and membrane stabilization

Experiment (g/mL)	STF-HAETR (µg/mL)				Aspirin
	% of protection				
	25	50	75	100	100
% of inhibition of protein denaturation	12.56 ± 0.57	12.56 ± 6.2	53.44 ± 5.45	75.67 ± 3.2	80.86 ± 1.56
% of membrane stabilization	21.56 ± 0.58	42.45 ± 6.2	62.32 ± 1.12	77.51 ± 3.43	65.47 ± 4.21
% proteinase inhibition	32.23 ± 5.67	46.35 ± 3.45	54.79 ± 5.56	67.47 ± 3.67	62.61 ± 4.45

Each value represents means ± SD ($n = 3$) in comparison to untreated controls

shown that IL-12 is very much required for the specific induction of IFN-γ production, for the activation of Th1 cells (Kim et al. 2000), so as per our in vitro PBMC cytokine assay results indicating the potent IL-12 suppression action of STF-HAETR might be also responsible for decreasing IFN-γ levels and subsequent protection against rheumatic arthritis and inflammatory disorders.

Protein misfolding due to generation of reactive oxygen species (ROS) during various inflammatory disorders leads to protein aggregates ultimately imparting an apoptotic action on surrounding tissue cells. During inflammation oxidative stresses, and formation of reactive nitrogenous intermediates (RNI) mainly by iNOS, together induce protein misfolding due to increased temperature and lowered pH on that site of inflammation. NO production from macrophages is also reported to be significantly up regulated by IFN-γ in inflammatory conditions (Belguendouz et al. 2011; Wyatt et al. 2014). As STF-HAETR showed a significant inhibition of iNOS expression levels leading to decrease of NO compared to controls, it may suppress the inflammatory mediators of IFN-γ. Overall; this could alleviate inflammation and rheumatic arthritis by STF-HAETR.

STF-HAETR remarkably inhibited in vitro protein denaturation with an alleviating effect on the proteinase activity of trypsin model. The in vitro anti-denaturation effect of STF-HAETR in BSA model, apparently suggests therapeutic role by protein stabilization during inflammatory responses like rheumatic arthritis (Saso et al. 2001). The in vitro trypsin inhibitory potentiality of STF-HAETR can control trypsin-mediated activation and release of inflammatory mediators through protease activated receptor-2, especially having a role in inflammatory processes in osteoarthritis pathogenesis (Miike et al. 2001). Damage of type II collagen at the surface of chondrocytes leads to a progressive degeneration of the cartilage during arthritis (Busso et al. 2001). Thus, proteinase inhibitory activity of STF-HAETR, might allow inhibition of the activity of collagenase enzymes in arthritic inflammatory lesions, resulting in suppression of tissue destruction and osteoarthritis disease progression.

Several researchers through histological studies showed that carrageenan-induced inflammation is linked to intense edema which is characterized by an increased level of migration and infiltration of inflammatory polymorphonuclear leukocytes (PMNs) cells, neutrophils, in the inflamed paw tissues. The in vitro human RBC membrane stabilization action of STF-HAETR against stressors of heat and hypo tonicity might be correlated with its ability to stabilize the analogous lysosomal and neutrophil membrane in inflammatory sites, thus preventing the release of lysosomal enzymes and other inflammatory mediators that could have augmented the inflammation. Stabilization of the membranes of polymorphonuclear neutrophils (PMN) leading to reduction of reactive oxygen intermediates is a potential ant-inflammatory mechanism of some of the anti-inflammatory drugs (Hussein et al. 2013). However, STF-HAETR's membrane stabilization property over human RBC against stressors like heat and tonicity could possess potentiality to stabilize PMN membrane, as RBC membrane is somewhat physiologically analogous to PMN membrane.

Our histamine bioassay in goat trachea model also revealed a significant anti-histaminic effect of STF-HAETR, as bronchoconstriction due to histamine was well prevented at a dose of 100 µg/mL. STF-HAETR's antagonistic effect on histamine, could further emphasis the possibility of restricted histaminic activity in case of inflammations especially in the initial phases of edematous paw inflammations when histamine plays a pivotal role in primary swelling, redness and pain (Tadesse et al. 2004).

Moreover, prevention of histamine mediated airway smooth muscle spasm in isolated trachea by STF-HAETR may represent a possible H-1 receptor mediated anti-asthmatic profile. In hypersensitivity associated allergic situations, degranulation of the IgE activated mast cells to releases histamine, prostaglandins, proteolytic enzymes, and cytokines, etc., trigger smooth muscle contractions. Cytokines like IL-6 and TNF-α released from mast cells along with T cells further initiates inflammation in asthmatic lungs (MacGlashan 2003; Bradding et al. 1994). STF-HAETR's action on down regulating pro-inflammatory cytokines and NO, and the in vitro inhibition of proteinase and protein denaturation along with a RBC membrane stabilizing activity could justify its use against o prevention of histamine induced asthmatic dyspnoea.

As our preliminary phytochemical analysis of tested root extract part STF-HAETR, indicated the presence of significant amounts of mainly two categories of constituents' steroids and terpenoids. It can be speculated that these ingredients might be highly responsible for the modified immunological effects and lesser toxicity than the earlier reported latex and aerial parts. Anti-inflammatory property of STF-HAETR as shown through its effect on multiple aspects of inflammatory cascades including down regulation of cytokines IL-12, TNF-α, IL-6 and decreased NO formation and potent COX-2 inhibition emphasizes it to be an effective anti-inflammatory drug. This potential analgesic, anti-inflammatory, anti-asthmatic and anti-arthritis effect may be executed may due to the presence of marker phytocompounds of β-sitosterol and lupeol (Table 2, shown in TLC profile of the fraction) as they were earlier established as powerful anti-inflammatory agents (Ku and Lin 2013). Though we have not assessed the pro-inflammatory cytokines levels and other inflammatory parameters in the treated rodents, the possible mechanism of action

could be delineated from our in vitro studies supporting the experimental clinical efficacy against pain, inflammation and asthmatic dyspnoea. Membrane stabilization property, protein denaturation prevention and trypsin (proteinase) inhibition, may enable STF-HAETR to impart additional effects on inflammatory sites by preventing local tissue destruction and integrity of cell membranes. Beside this, the anti-histaminic effect of STF-HAETR further potentiates its ability to dampen inflammatory responses in a broader way. Additional biological study is warranted with active moiety isolation against asthma and, arthritis, etc., so that to achieve a successful drug candidate.

Our primary objective was to establish the potentiality of total steroid and terpenoid fraction of HAETR as a potent anti-inflammatory with a robust mode of action upon inflammatory cascades of pro-inflammatory cytokines, COX and other mediators. Thus, we assessed the test fraction in carrageenan paw edema model, as it is a well validated model which elicits a systemic inflammation with a substantial elevation of proinflammatory cytokines, NO production, etc. (Vazquez 2015). Moreover, it serves as a classical model for screening anti-arthritics, NSAIDs, although it resembles the acute inflammatory state, but the etiological parameters are very much similar in contrast to the inflammation associated with osteoarthritis (Sharma et al. 2004).

But models like collagen and CFA induced arthritis typically elucidate autoimmune types or rheumatoid arthritis, which was not our primary objective even though in future we will assess upon this models for preventing chronic inflammatory disorders like rheumatoid arthritis.

Conclusion

This study concluded that the bioactive fraction of STF-HAETR reduced pain by selective COX-2 inhibition and attenuated all kinds of inflammatory syndromes by down regulating NO production through decreasing iNOS expression level, TNF-α, IL-6 and IL-12 cytokine levels. The anti-inflammatory and anti-nociceptive properties were reflected in vivo model of carrageenan-induced acute inflammatory paw oedema and acetic acid induced peripheral and hot plate induced central nociceptive pain model without any untoward effects. It reduced the in vitro asthmatic complications by dilating histamine induced bronchoconstrictions. STF-HAETR significantly improved arthritic status by protecting protein denaturation via proteinase inhibition. Therefore, it is worthy in mentioning that robust anti-inflammatory property of total steroid–terpenoid enriched fractions of *Euphorbia tirucalli* could be explored in various inflammatory and immune deregulated conditions like asthma, arthritis and nociceptive pain as potential drug candidate.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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Chapter 14

Role of Stress in Diseases and Its Remedial Approach by Herbal and Natural Products in Stress-Related Disease Management: Experimental Studies and Clinical Reports

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In today's 21st century there is a huge generational shift in terms of socio-economic, work, and cultural pressures that can lead to a stressful life. Also there has been a subsequent rise in the range of disorders affecting major populations regarding health and monetary consequences [1]. Stress affects practically everybody on the planet from schoolchildren to the elderly population. The role of modern medical science is constantly updating its parameters to understand the causes of psychological stress-induced disease and its treatment. However, in many cases of chronic lifestyle diseases, one significant point regarding the prognosis of patients is that treatment is focused only on the apparent pathophysiological aspect of the disease, not upon the grassroot level or the underline hidden cause, such as "psychological stress," which remains often unaddressed. As a precursor to many diseases, psychological stress may also worsen in patients suffering from several chronic diseases [2]. According to the World Health Organization and the Global Burden of Disease Survey, it has been estimated that mental disease, including stress-related disorders, will be the second leading cause of disabilities by the year 2020 [3]. Moreover, the United Nations in 1992 affirmed that stress was a 20th

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century disease. Also a proposal has been made to include stress-associated mental disorders and possible outcomes in International Classification of Diseases-11 [4]. The American Psychological Association stated in a survey report that teenagers and younger children are more stressed than adults [5]. In 2011 the Nielsen survey of 6500 women in 21 countries conferred a report showing that women are the most stressed on earth, among them Indian women rated at 87%, which is the highest in the world [6].

Stress is a part of everyday life, which consists of an individual perceptual phenomenon that primarily originates from the imbalance between demand on the individual and his or her ability to cope. Stress is not an illness, rather it is a state. If stress becomes too excessive and prolonged, mental and physical illness may develop. Stress is an adaptive response of the body to psychological and physiological stressors, comprising factors leading to disruption of the normal homeostasis of the body [7]. Physiological stressors are infection, severe illness, fever, blood loss, etc., whereas associated depression, anxiety, fear psychosis, tension due to prime triggering factors such as unemployment, work load, job insecurity, traumatic incidence, family problems, conjugal-life problems, being abused or neglected, conflicts in interpersonal relationships, losing contact with loved ones, etc. are negative personal stressors that lead to psychological stress [8]. Recurrent intense stressful situations are detrimental to the body, resulting in psychosomatic disturbances, which in future may turn into organic illnesses as the responses become pathological in nature [9]. Psychological stress now affects millions of people globally, ultimately leading to increases in health adverse effects such as cognitive and behavioral diseases, heart diseases, metabolic syndrome, infertility, immune problems, psychosomatic disorders, and related neurotic disorders. Several studies reveal that stress reductions in cases of heart diseases, hypertension, diabetes, infertility, etc. give very promising effects on disease progression [10].

1. PATHOPHYSIOLOGY OF STRESS RESPONSE

Stress affects two major body systems: the hormonal system, which is the hypothalamic−pituitary−adrenal (HPA) axis, and the sympathetic adrenomedullary system (SAS). Stress leads to secretion of corticotrophin-releasing hormone (CRH) from the hypothalamus, and then CRH acts on the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). Finally, ACTH acts on the adrenal cortex to release cortisol and epinephrine [11]. Thus activation of the HPA axis ultimately leads to elevated levels of plasma cortisol, a glucocorticoid and end product of the HPA axis that is secreted within a stable and narrow range through the tight regulation of a feedback system that checks excessive and sustained cortisol secretion. Thus a normal response of the HPA axis to stress and subsequent cortisol secretion is maintained by a feedback system, circadian variation, and central regulation. The normal pattern of ACTH and cortisol secretion reaches its peak plasma level in the early morning, decreases during the day, and becomes lowest at

midnight. However, at times of chronic exposure to stress, disruption of the HPA axis occurs, which is characterized by downregulation of glucocorticoid receptors in the hippocampus and pituitary gland, causing an increased CRH and cortisol response to stress with inappropriate feedback regulation resulting in disturbed normal diurnal cortisol rhythm [12]. Simultaneously, hyper-activation of SAS results in the release of norepinephrine and epinephrine with an inhibitory effect on GABAergic transmission. The released high concentration of cortisol increases the synthesis of adrenaline from noradrenaline in the adrenal medullary cells through induction of the methylating enzyme [13]. Furthermore, cortisol inhibits extraneuronal uptake of adrenaline by inhibiting the extraneuronal amine transporter and organic cation transporter, thus potentiating further excitatory adrenergic transmission in stressful situations (Fig. 14.1) [14].

In emergency situations or because of acute stressors such as infections or preexams, stress is essential or to some extent physiological. However, chronic and constant hyperactivation of the HPA axis mainly in psychological chronic stress alters the normal homeostatic balance of the body leading to neuro-hormonal imbalances. In normal conditions in the body, excitatory signaling is counterbalanced by inhibitory signaling, which results in activation of compensatory adaptive responses leading to reestablishment of homeostasis and protection against stress. This stress system is vital in homeostasis maintenance. However, under chronic stressful situations, inhibitory signaling is not able to counterbalance excitatory signaling efficiently leading to chronic and continuous activation of the HPA axis and SAS. This can be manifested by chronic elevation of blood pressure (BP), change in heart rate (HR), etc.,
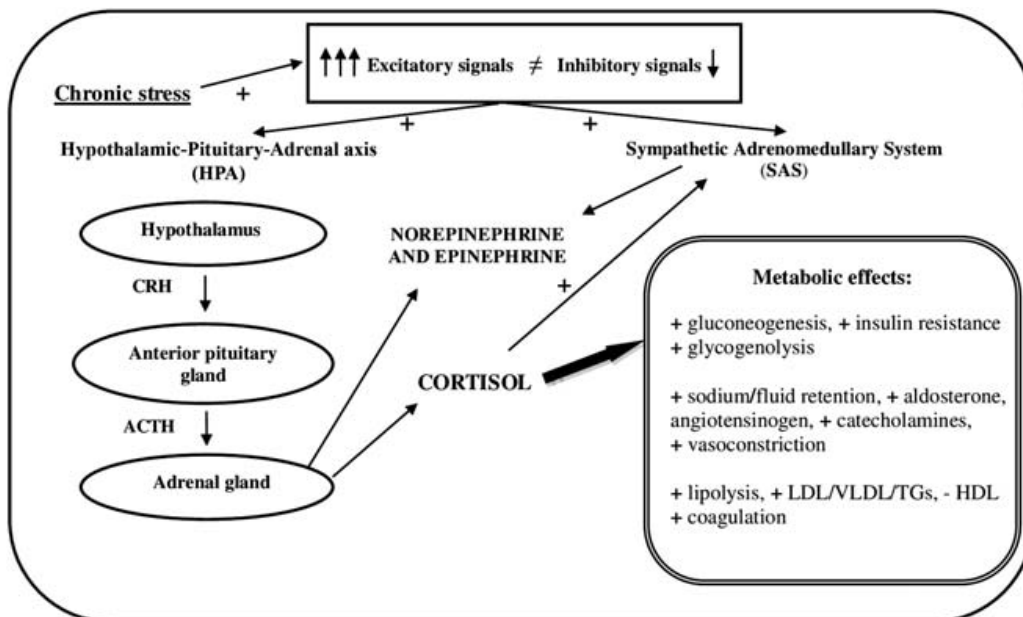

FIGURE 14.1 Physiological stress response in diseased conditions. *HDL*, High-density lipo-protein; *LDL*, low-density lipoprotein; *TGs*, triglycerides; *VLDL*, very low-density lipoprotein.

which affects various systems of the body such as the cardiovascular system, central nervous system (CNS), gastrointestinal (GI) system, immune system, cognitive function, growth and reproductive axis, etc. In the long run these lead to various stress-related disorders, including cardiovascular, metabolic, behavioral, endocrine, autoimmune, etc. disorders [15]. Ultimately, psychological stress disturbs the quality of life and a growing number of illnesses have been found to be associated with it [16]. Apart from stress-induced diseases, patients suffering from chronic diseases are also much more stressed because of their illness, medications, treatment cost, etc., which ultimately hastens their recovery from primary treatment. Patients suffering from metabolic syndrome and heart diseases who are under standard medicine treatment often require dose escalation and additional drugs in the long run because chronic stress-related elevated cortisol levels are poorly managed or neglected. So, a novel antistress product devoid of any side effects may become a gold standard treatment in cases of stress-associated diseases, which will prevent further disease complications.

Long-term psychological stress results in the manifestation of symptoms, mainly:

1. Cognitive, behavioral, and emotional symptoms: memory problems, poor concentration, anxiety, nervousness, negative thoughts, irritability, depression, eating more or less, sleep disturbances, addiction to smoking and drinking, nervous habits such as nail biting, etc. [17].
2. Physical symptoms: back ache, muscle tension, GI disturbances (diarrhea, constipation), nausea, rapid HR, frequent colds, loss of libido, etc.

2. IMPACT OF PSYCHOLOGICAL STRESS ON OCCURRENCE OF DISEASES

2.1 Cardiovascular Diseases

Clinical data points to the fact that long-term psychological stress is associated with increased risk of coronary heart disease (CHD) [18]. The INTERHEART study is the largest study conducted for evaluating the risk factors for myocardial infarctions in over 262 centers in 52 countries. The study included 11,119 patients suffering their first myocardial infarction and 13,648 age-matched and sex-matched controls. Ultimately, very surprising conclusions showed that out of the nine risk factors, psychological stress was the third most prevalent risk factor associated with acute myocardial infarction, after apolipoprotein B/apolipoprotein A ratio and smoking [19]. Interestingly, diabetes and hypertension were lesser risk factors than psychological stress. Furthermore, the Whitehall II prospective cohort study of over 10,308 civil service employees after a 5-year follow-up concluded that psychological stress was associated with an increased incidence of CHD and electrocardiogram

abnormalities [20]. Another study of 514 healthy men and women depicted that after experiencing mental stress through behavioral tasks, salivary cortisol levels were elevated and significantly associated with coronary artery calcification (CAC) [21]. However, only 40% of the participants were cortisol responders after stress induction, which reflects the variation in the stress-coping capability of different individuals. Reports showed that elevated urinary levels of the stress hormone cortisol in 861 participants aged 65 year and older suggested that they had a five times increased risk of dying of cardiovascular disease [22]. Elevated cortisol level, which is a biomarker for stress, is related to atherosclerosis of the carotid arteries and is associated with a history of cardiovascular disease [23].

There is evidence that a glucocorticoid receptor gene is related to high proinflammatory activity and CHD risk. It has been shown that blocking cortisol activity prevents stress-induced endothelial dysfunction and baroreflex impairment [24]. A population-based prospective study found an association between cortisol and incident ischemic heart disease, which reflects the effects of chronic stress and behavioral factors [25]. The study also revealed that each change of 10 mmHg in systolic BP in young adults during a video game stressor was associated with increased risk of having CAC in relation to poststress cortisol elevation after 11 years of follow-up [26]. Heightened mental stress response also predicts the possibility of future hypertension, increased low-density lipoprotein/very low-density lipoprotein (LDL/VLDL) triglyceride levels, and subclinical cardiovascular disease progression [27]. Coagulation activity is increased very efficiently through a complex mechanism by cortisol and catecholamines, accompanying plaque rupture and thrombosis leading to future cardiovascular events. Additionally, cortisol has an action on myocardial contractility and tone of arterioles. Circulating elevated cortisol levels are also the initiator of perivascular inflammation and calcification within arteriosclerotic lesions [28].

2.2 Hypertension

Abnormality of the HPA axis and SAS system by psychosocial stress is one of the reasons for the development of hypertension. Studies also indicate that high levels of stress in prehypertensive men were associated with increased risk of progressing to hypertension and incidence of CHD. In a study of 489 healthy normotensive men and women, salivary cortisol levels were measured after facing a 5-min Stroop test (physiological stress) and mirror tracing test (psychological stress). After a 3-year follow-up, the sample that responded to elevated cortisol developed hypertension, suggesting association between psychological stress cortisol reactivity and incidental hypertension [29]. Extensive research indicating the point at which participants were at risk of hypertension showed heightened HPA activity in response to acute stressors. Furthermore, cortisol has a permissive effect in potentiating the pressor

activity of adrenaline and angiotensin by direct and indirect effects during simultaneous epinephrine release by SAS activation resulting in vasoconstriction and increased HR [30]. Glucocorticoid receptors present in the heart, smooth muscle cells of the resistance vessels, and the kidneys directly affect BP. Furthermore, cortisol enhances angiotensinogen formation, a substrate of rennin resulting in increased angiotensin II levels, and subsequently affects the rennin—angiotensin system, which is a vital physiological system in maintaining blood volume and electrolyte status in relation to BP regulation [31]. Aldosterone and vasopressin syntheses are also augmented, affecting sodium and fluid retention causing sustained increase in BP due to vascular overload [32]. There is also research regarding the molecular actions of cortisol in the inhibition of vasodilation via decreased levels of plasma kallikrein, prostacyclin, and nitric oxide synthase (NOS) activity [33]. Increased expression of adrenergic and AT1 receptors in vascular smooth muscle cells by chronic elevated cortisol levels is another pathway to BP elevation [34].

2.3 Diabetes

There has been a positive correlation among psychological stress and the incidence of type 2 diabetes mellitus (DM) according to a large number of clinical reports. A major prospective MONICA/KORA Augsburg study declared some interesting outcomes over 5337 of the working population aged from 29 to 66 years without any history of diabetes. After a follow-up of 12.7 years, 291 cases of type 2 DM were observed and participants with high stress levels due to job strain had a 45% higher chance of developing type 2DM than those with a low job strain [35]. So, a high job strain with mental stressors is a vital precursor to type 2 DM independently of traditional risk factors. A relation can be drawn between increasing cases of diabetes among the young adult population and the burden of psychosocial stressors such as career, work load, relationship complexities, etc.

Cortisol and catecholamines, the end products of stress, to some extent have similar metabolic effects. They alter the glucose metabolic pathway by using a plethora of innervating effects. Cortisol promotes glycogen deposition in the liver by inducing hepatic glycogen synthase; it also promotes gluconeogenesis and glycogenolysis in the liver [36]. It inhibits glucose utilization by the peripheral tissues and causes decreased glucose uptake in skeletal muscle [37]. The catabolic action of cortisol causes protein breakdown, and amino acid mobilization from peripheral tissues is used up in gluconeogenesis resulting in increased urea production with negative nitrogen balance in the body. This along with increased glucose release in the liver results in hyperglycemia and causes insulin resistance and a diabetes-like state. In addition, increased adrenergic activity due to SAS activation induces downregulation of insulin receptors. Lipolysis is also stimulated by cortisol, epinephrine, and norepinephrine resulting in increased plasma free fatty acids, which further

inhibit release of insulin in response to glucose. Thus all these effects converge to create further insulin resistance and glucose intolerance-like states [38]. Beside chronic stress as a precursor of diabetes, it has been implicated that the further existence of psychological stress in patients with existing type 2 DM results in poor glucose control and future associated complications such as nephropathy, neuropathy, macroangiopathy, etc., which are directly correlated with cortisol secretion [39]. Experimental acute psychological stress is reported to increase postprandial glucose concentrations significantly in patients with type 2 DM, which further justifies the need for stress management in diabetic patients [40].

2.4 Metabolic Syndrome

Metabolic syndrome is characterized by the coexistence of hypertension, diabetes, hyperlipidemia, and abdominal obesity in an individual state, which is recognized as an important risk factor for CHD and mortality. Psychological stress related to depression or environmental stressors is associated with increased cortisol levels, which predispose to features of metabolic syndrome [41]. In a double-blind case−control study of 10,308 people it was found that working people aged between 35 and 55 years with metabolic syndrome had greater psychological stress associated with higher cortisol levels and epinephrine activity compared to healthy controls [42].

As discussed earlier regarding the roles of stress hormones in progression to hypertension and diabetes, there is a prevalence of insulin resistance, which clusters together with increased LDL, VLDL, and triglycerides due to altered lipid metabolism, ultimately leading to features of metabolic syndrome. Insulin resistance is thought to be the underlying abnormality that causes metabolic syndrome-associated complications. Stressor-like depression also predicts insulin resistance, diabetes, and metabolic syndrome [43]. Obesity is a major problem; in fact, visceral obesity, which is a feature of metabolic syndrome, is nowadays found to be very common. People who are greatly exposed to mental stressors such as low social status, anxiety, exhaustion, etc. have more visceral obesity or intraabdominal fat accumulation and constitute dysregulation of cortisol circadian pattern [44]. Stress-induced excess cortisol increases growth and functions of visceral fat cells leading to abdominal adipose tissue accumulation, whereas released catecholamine during SAS activation stimulates inflammatory adipocytokine production such as interleukin (IL)-6 in adipose tissue [45]. This inflammatory cytokine along with increased angiotensin II levels contributes to insulin resistance, thus metabolic syndrome status begins [46].

2.5 Stroke

Research has confirmed that leading a stressful life due to psychological stressors resulting in behavioral symptoms of anxiety, apathy, etc. significantly

increases the risk of incidental stroke in humans [47]. In a study among 6749 human subjects, researchers implicated that those who reported higher levels of mental stress were at the greatest risk for having a stroke, compared to those with lower levels of psychological stress and depression [48]. In that particular study, psychological stress significantly increased transient ischemic attack risk by 59%, which is a ministroke, caused by temporary blockage of blood flow to the brain. Mental stress can be related to an increased risk of cerebrovascular disease, as excessive sympathetic activity occurs during stress-induced SAS activation leading to vasoconstriction. The mechanism of chronic psychosocial stress affecting the vascular system, as discussed earlier under hypertension, can also be attributed to the sustained pressor effects of elevated cortisol by potentiating adrenaline and angiotensin levels and lowering plasma kallikrein, prostacyclin, and NOS in vascular endothelium leading to inhibition of vasodilation and ultimately decreased blood flow. Also increased coagulation, vascular calcification, and inflammation are triggered by both cortisol and catecholamines released in stress, which can also be a possible mechanism for strokes.

2.6 Infertility

Perceived mental stress has been regarded as detrimental to successful human reproduction, since a lot of reports are showing a relation between stress and unsuccessful pregnancy outcomes. Data show that psychological stress significantly reduces the probability of conception each day during the fertile window in women [49]. Human data show that chronic stress causes dysmenorrhea and anovulation in women, and lowers sperm count and sperm motility in men [50]. In a study among men from infertile couples, higher amounts of cortisol levels were found in the group of men with low sperm count compared to the control group with normal counts [51]. Couples who have tried to conceive for a longer time always experience frustration and distress if pregnancy is not achieved. Also in infertile couples, much higher levels of psychological stress have been reported among women than men [52]. Stress affects both male and female fertility by causing neurohormonal imbalance due to disruption of normal HPA axis-mediated cortisol secretion. Increased levels of cortisol inhibit the gonadal axis by inhibiting secretion of gonadotropin-releasing hormone (GnRH) resulting in decreased levels of follicular stimulating hormone (FSH) and leutinizing hormone (LH), ultimately causing impaired ovarian and testicular functions [53]. Due to this serum testosterone, estrogen and progesterone levels are lowered and lead to fertility problems. In human males, severe psychological stress causes decreased testosterone synthesis by Leydig cells, which is also due to cortisol-induced apoptosis of Leydig cells [54]. Furthermore, in females, cortisol hampers uterine growth and differentiation [55]. It has been postulated that stress-mediated overactivation of sympathetic nervous transmission into

excess released catecholamines alters blood flow in the fallopian tubes and gamete transportation through adrenergic receptors present in the reproductive tract [56].

Ultimately, couples who suffer from infertility frequently turn to artificial reproductive technology (ART) such as in vitro fertilization (IVF), which has increased by 83% [57]. It is required to measure mental stress levels before and during fertilization treatment to achieve success in pregnancy. Studies have shown that psychological stress significantly reduces the chances of obtaining pregnancy with the IVF procedure. Before IVF treatment, women who had mental stress factors such as concerns about aspects of the procedure, treatment cost, etc. had lower pregnancy outcomes after IVF compared to those who were not stressed [58]. Stressful behavior reduces fertilization, implantation, and live birth rates, thus affecting IVF outcomes [59]. Impaired ovarian function due to estradiol inhibition by the stress hormone cortisol leads to decreased number of oocytes to be harvested in IVF [60]. Interruption of menstrual cycle due to mental stress, which is very well documented, is also a causative factor for infertility and unsuccessful IVF outcomes [61].

2.7 Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is the most common gynecological disorder, affecting 12% women of reproductive age with features such as acne due to hyperandrogenism, hirsutism, obesity, irregular menstruation, and anovulation, and is ultimately the leading cause of infertility in women [62]. There is a prevalence of higher levels of behavioral and psychological stresses in patients suffering from PCOS [63]. Reports suggests that PCOS patients have higher risks for developing CHD and type 2 DM (eightfold increased chance), which are manifestations of insulin resistance in metabolic syndrome, thus connection of psychological stress is well established [64].

A study reported that public speaking in PCOS patients resulted in higher plasma ACTH and cortisol levels, with an increased HR and SNS activation compared to normal healthy individuals [65]. Furthermore, they showed that increased levels of baseline serum IL-6 levels in PCOS patients over controls was an indication of the mechanism behind stress-related insulin resistance. Other clinical studies also report higher amounts of perceived mental stress, cortisol levels, and higher HPA axis activation in PCOS women over normal individuals [66]. Thus besides conventional hormonal treatments, PCOS treatment should also cover psychological aspects such as stress reduction to increase the outcomes of infertility, hyperandrogenism, etc..

2.8 Pregnancy Outcomes and Miscarriages

In the previous section the adverse role of psychological stress in causing infertility, menstruation problems, PCOS, etc. was mentioned, but the role of

the gestation period ultimately leading to birth defects, miscarriages, etc. has to be discussed. Psychological stress in pregnant women has been clinically established as a vital risk factor for spontaneous abortions [67]. An observational study of pregnant woman confirmed that of those who had increased stress levels with elevated urinary cortisol, 90% resulted in spontaneous abortion, i.e., very early pregnancy loss [68]. As discussed earlier, stressful behavior is related to increased cortisol and decreased FSH, LH, and GnRH release; additionally, cortisol affects luteal progesterone production, and combines to affect uterine maturation and pregnancy maintenance [69]. Studies in humans also confirm the role of maternal psychosocial stress in shortening of the gestation period, which results in preterm delivery and premature babies [70]. So, maternal stress reduction is vital for successful pregnancies and neonatal health.

2.9 Gastric Ulcer

Stress ulcers are commonly encountered in clinical practices, in which mental and behavioral factor-associated stress is greatly responsible. A human observational study indicated the positive association of higher incidence of gastric ulcer in subjects with higher amounts of psychological distress [71]. Cortisol increases the secretion of pepsin and gastric acid in the stomach [72]. Moreover, cortisol decreases the formation of prostaglandins, which are gastroprotective, through negative regulation of the expression of COX-2 [73]. Ulcer-causing effects can also be delineated by cortisol-mediated induction of lipocortins, which are inhibitors of phospholipase A2 and thus result in decreased prostaglandin production [74].

2.10 Irritable Bowel Syndrome

Enhanced mental stress response has been found to be a potential mechanism for pathophysiological changes in causing irritable bowel syndrome (IBS) due to dysregulation of HPA axis activity [75]. A number of studies have depicted that IBS patients perceive greater psychological stress with higher levels of plasma cortisol over healthy controls. In postexperimental mental stress in IBS patients there are also reports of escalation in problematic gastrointestinal symptoms, which also might further establish the relation between perceived stress and the severity of IBS [76]. Crohn's disease and IBS patients have higher anxiety states, depression, and mental exhaustion as reported through a specific stress questionnaire [77]. Research in IBS patients also indicates that a psychological stressor such as public speaking hampers intestinal permeability of IBS patients, and mast cell degranulation is positively correlated with increased salivary cortisol levels [78]. Also research in healthy young women established that increased exposure to psychological stress resulted in

prolonged mucosal dysfunction in the jejunum in response to stimuli, which may also represent an initial step in the development of IBS [79].

2.11 Osteoporosis

In a study, depressed young men and women with increased basal cortisol levels, who were without any signs of osteoporosis, were observed for a number of years. Ultimately, observation concluded the significant occurrence of osteoporosis in the depressed subjects, which was also found to be positively associated with elevated cortisol levels [80]. The possible explanation for stress-mediated progression to osteoporosis can be made through cortisol's inhibition of intestinal calcium absorption, with heightened renal calcium excretion, which ultimately leads to calcium loss from bones and decreased osteoid formation and resorption [81].

2.12 Decreased Immunity and Delayed Wound Healing

A number of extensive clinical reports have suggested that mental stress, emotional behavior, etc. decrease the immunity of the body and thereby make the body prone to easily infectious diseases. Reports indicate higher incidences of infectious diseases such as respiratory tract infections, urinary infections, etc. and also neoplastic disease in chronic mentally stressed persons [82]. Clinical cough and cold are frequently reported as common incidents in psychologically stressed students who possess large social networks [83]. A study among medical students revealed that there was significant increase in plasma cortisol levels with a marked decreasing effect on lymphocyte proliferation, IL-2, and lymphocyte CD19 production on the day before examination compared to the beginning of the academic year [84].

Cortisol, which is a glucocorticoid, has a profound pharmacologically immunosuppressive effect through wider suppression of major inflammatory responses. Stress-influencing immunity is attributed to the actions of cortisol, which has a lympholytic action, suppression of the proliferation of T-lymphocyte, and higher rate of destruction of lymphoid cells [85]. Cortisol downregulates the production of IL-1, IL-2, IL-3, tumor necrosis factor-α, and γ-interferon by negative regulation of genes for cytokines in macrophages, endothelial cells, and lymphocytes, resulting in interference of chemotaxis [86]. Cortisol has also been found to decrease the production of cell adhesion molecules such as ELAM-1 and ICAM-1, which affects decreased adhesion and localization of leukocytes in wound healing [87]. Further decrease in the production of acute phase reactants from macrophage and endothelial cells results in interference of the complement function. Thus cortisol favors the spread of infection in the body as the capacity of microorganism killing by defensive immune cells is impaired.

In human subjects it has been established that psychological stress also impairs wound healing, which can reflect poor recovery from surgery [88]. Study also reflects that during examination stress in a group of students, experimentally induced wounds healed after a much longer time when compared to the control ones at summer vacation time [89]. Further studies in humans also clearly show the positive correlation between psychological stress-induced elevated cortisol levels and decreased rate of wound healing [90].

2.13 Mental Diseases

In young insomniac patients, elevated cortisol secretion patterns along with increased sympathetic activity are well established. Chronic stressful events leading to HPA overactivity in insomnia through significant elevated cortisol levels suggests that insomniacs are at risk for anxiety, depression, and psychosis with significant morbidity [91]. Chronic elevated cortisol levels are associated with stressful situations on a daily basis, which ultimately progress to depression and psychosis in the long term, especially in subjects who have impaired coping ability due to impaired HPA axis functioning. Cortisol has been found to increase the expression of serotonin reuptake transporter in humans resulting in higher serotonin uptake, thus directly leading to clinical depression [92]. In a metaanalysis, augmentation of cortisol levels was much higher in clinically depressed persons compared to nondepressed subjects in response to an experimental psychological stressor [93]. Findings also indicated a positive relation between daily life stressors as risk factors for the onset of psychotic symptoms in adolescents. Moreover, an analysis over a baseline and 1 year follow-up among psychotic youths indicated that those who developed psychosis showed significantly higher salivary cortisol levels at the baseline during the first follow-up [94]. Moreover, psychological stress also victimizes short-term memory and increases forgettability to a greater extent. Significant findings have related that the decrease in cortisol levels in the humorous mood of elderly individuals while watching a humorous video caused a greater improvement in learning ability and memory recall [95].

2.14 Need for Herbal and Natural Drugs in the Management of Psychological Stress

Psychological stress management is urgently needed to prevent stress-induced diseases and to increase the quality of life and productivity. However, there are a number of lifestyle modifications and nonpharmacological approaches such as meditation, exercise, music, etc. that may increase the body's adaptation to stress. Nonetheless, shortcomings of this therapy are nonspecific, with fewer clinically proven efficacies, variable acceptances, and questionable approaches and qualities. Speaking of pharmacological approaches, in the modern allopathic system of medicine, antianxiety agents of the benzodiazepines class

such as diazepam and antidepressants such as selective serotonin reuptake inhibitors (selective seretonin reuptake inhibitors [SSRIs]) are often prescribed for frank stress management. Though they acutely alleviate some of the behavioral symptoms by affecting GABA transmission and other central neurotransmissions in the body, chronic sustained elevated cortisol by HPA axis involvement remains unaddressed. These synthetic pills also have drawbacks, e.g., they are meant for short-term usage only and there is a risk of dependence, hangover, withdrawal symptoms, cognitive impairments, etc. So far there has been no allopathic or synthetic drug that is specific for antistress situations, which without any adverse effects can safely prevent the elevation of stress-induced cortisol. Today, herbal drug research has reached its peak in terms of active moiety isolation, preclinical studies, and also human clinical trials. As mentioned earlier, some of the potential antistress compounds as isolated molecules with structure elucidation as per modern herbal drug discovery seek genuine standardization of the antistress herbs for therapeutic utility and efficacy. Thousands of years of traditional concepts from Ayurveda, Chinese traditional medicine, etc. have documented valuable herbs for respective vivid indications, which can act as a vital hub for modern drug discovery research. In relation to the management of psychological stress, "adaptogens" that improve an individual's coping ability to stress is also a complex and new drug research area. In situations of increased stress these herbs normalize the physiological process of the body and increase the ability of an organism to adapt to environmental factors and prevent damage from stressors.

Ideally, an antistress candidate should significantly prevent chronic psychological stress-induced cortisol elevation because cortisol is a validated stress biomarker, which seems to be the ideal target for prevention of stress-induced disorders. It should not interfere with normal circadian rhythmic secretion of ACTH, and should only prevent excess sustained cortisol release as in mental stress-associated impaired cortisol release rhythm. Besides this the drug should not impose any adverse effect on the HPA axis, should be free from withdrawal syndromes, and should possess its beneficial effect even if the dosing is tapered. In animal models of stress, extensive study has been done with natural-based compounds, but establishment of an exact replica of human psychological stress in animals is not very practical as compared to controlled human studies. A large number of clinical trials with herbal-based products have been conducted on volunteers for assessing their antistress therapeutic potential, through validation of psychological stress monitoring by mental tasks, computer games, questionnaires, etc. Many of them have shown quite promising effects in human trials by significantly lowering experimental stress-induced plasma and salivary cortisol levels with insignificant side effects. The following are some herbal and natural products that are clinically proven antistress discoveries in human clinical trials.

3. HERBAL THERAPY

3.1 *Withania somnifera*

This is also known as "ashwagandha." It is mentioned in Ayurveda for having rejuvenating power and stamina. The roots of the herb possess clinically and therapeutically confirmed antistress properties as shown in a number of human trials. A double-blind, randomized, and placebo-controlled trial with 64 adults, chronically suffering from mental stress, at a dosage 300 mg of ashwagandha root extract twice a day for 60 days, showed promising results [96]. In that study, stress scores were assessed by using numerous, well-recognized, globally accepted stress questionnaires and a serum cortisol estimation on days 0 and 60. The study concluded that chronic ashwagandha ingestion was significantly responsible for lowering the stress score and serum cortisol levels over placebo and baseline at day 0, with no significant reports of adverse effects during the treatment period.

Several animal studies also validate its clinical utility by significantly ameliorating chronic stress-triggered pathologies such as hyperglycemia, cortisol elevation, immunosuppression, gastric ulceration, and cognitive deficits in chronic foot shock-induced stress models [97]. Withanolides is one of its active constituents, which has been found to be responsible for its adaptogenic activity through improving brain oxidative status in animal models of chronic stress [98]. Another novel withanolide-free aqueous fraction of the root possesses antistress efficacy with a high therapeutic index in mice models, which might indicate other responsible polar antistress compounds [99,100]. The herb is also reported to increase anabolic activity and mice swimming endurance activity, thus nullifying stress-related fatigue [101].

In another double-blind, randomized human study, the antistress properties of standardized ashwagandha root extract were established in a group of mentally stressed subjects. In that study, ashwagandha intake for 2 month significantly prevented the elevation of serum cortisol, serum C-reactive protein, pulse rate, and BP over placebo controls. Improvement was reported in stress-associated symptoms such as fatigue, loss of appetite, headache, palpitations, sleeplessness, irritability, etc. through a Hamilton anxiety scale questionnaire [102]. In relation to therapeutic management of male infertility due to psychological stress, a study result supported the use of 3 g of herb per day for 3 months, as it is reported to increase fertility in normozoospermic psychologically stressed persons [103]. In that particular clinical study, ashwagandha significantly lowered plasma cortisol levels in psychologically stressed infertile men, and there were significant increases in fertility rate, sperm motility, semen quality, and LH levels over untreated individuals. Thus ashwagandha seems to be an effective antistress drug having a therapeutic prospect in managing psychologically stress-induced infertility.

3.2 *Panax ginseng*

Ginseng is an excellent adaptogenic ancient Chinese herb, which contains mainly ginsenosides as bioactive constituents. Ginsenosides are the most active therapeutic moieties possessing antistress properties, as per the reports of various animal studies of acute and chronic stress-induced pathologies affecting metabolism and immune and hormonal status [97,104,105]. Ginsenoside's neuromodulating effects have been found to prevent a chronic stress-mediated decrease in brain-derived neurotropic factor levels in mice, especially in the hippocampus region [106,107]. It is also a therapeutically established antistress herb that has been validated through a number of clinical trials in stressed human beings. In a study among postmenopausal women suffering from long-term psychological stress having symptoms of fatigue, insomnia, depression, etc., ginseng intake for 30 days resulted in a startling improvement as per the results of psychological stress assessments such as the Cornell Medical Index and the State-Trait Anxiety Inventory indicate. Postmenopausal women showed a higher stress score with serum cortisol elevation, but after treatment with ginseng at the 30th day the stress score was in the normal range with a significant decrease in serum cortisol concentration [108].

3.3 *Eleutherococcus senticosus*

This is also known as Siberian ginseng, having similar adaptogenic properties to *P. ginseng*. Its root and stem bark has traditional usage in China, Korea, and Japan, claiming to relieve fatigue and increase vitality. Eleutheroside E is an iridoid glycoside isolated from *E. senticosus* extract and is responsible for its antifatigue property, as evident from various animal studies [109, 110]. Animal data reflect that forced swimming, stress-induced immune downregulation and cortisol elevation are very much inhibited by Eleutheroside E administration [111], which is also evident in human studies. Furthermore, Eleutheroside E also has the propensity to prevent behavioral alterations and cognitive deficits due to stress induced by sleep deprivation in mice [112].

   In a clinical trial on a group of club-level endurance athletes under training, which served as a severe stressful situation, *E. senticosus* consumption for 6 weeks was related to lower values of serum cortisol, as reported after posttraining compared to the placebo group [113].

3.4 *Magnolia officinalis* and *Phellodendron amurense* Combination

Magnolia and Phellodendron standardized bark extracts are clinically validated as antistress in a combination form, as various human trials indicate. *M. officinalis* and *P. amurense* combination (MPC) treatment in 56 moderately stressed patients for 4 weeks demonstrated a significant reduction of salivary cortisol levels with an improvement in mood state parameters of anger,

tension, confusion, fatigue, etc. in comparison to the placebo group [114]. Among premenopausal women suffering from psychological stress, 6 weeks of oral MPC, extract treatment effectively reduced stress-perceived scores with broader aspects of mood symptoms associated with postmenopausal condition [115]. Magnolol and honokiol are the reported phytomolecules present in magnolia bark, which are being found to interact with the benzodiazepine site of the GABA(A) receptor, which might be responsible for exerting its effect upon controlling anxious and stressful behavior [116,117].

Another human study also indicated the cortisol and stress score-lowering property of MPC upon healthy overweight psychologically stressed women. This study also reported that the extract is free of adverse effects at a dose of 750 mg per day for a period of 6 weeks [118]. Furthermore, in pilot studies in humans, placebo-controlled trial data also suggest the effectiveness of MPC treatment on lowering perceived stress symptoms and cortisol levels, with additional decreased weight gain levels in overweight premenopausal females [119].

3.5 *Rhodiola rosea*

This is a well-known herb in European and Asiatic traditional medicine, widely used for depression, fatigue, stress, etc. since ancient times. Rosavin and salidroside isolated from this plant are largely responsible for its antistress efficacy in both acute and chronic stress models in rodents [120,121]. Salidroside and *R. rosea* extracts are simultaneously reported to decrease the levels of phosphorylated stress-activated protein kinase in immobilized stressed animals, with a modulatory effect in nitric oxide and cortisol release [122].

In a double-blind, randomized, and placebo-controlled study, *R. rosea* extract for 20 days to a group of mentally stressed students during an examination period showed a significant improvement in mental and physical symptoms associated with examination stress compared to placebo treatment [123]. Another clinical study among 161 young subjects under exposure to acute mental stress also showed that *R. rosea* treatment ameliorates stress symptoms such as fatigue, anxiety, etc. with a dual reduction in the physical parameters of BP and pulse rate [124]. Furthermore, another double-blind study showed that 28 days of *R. rosea* extract-repeated intake in psychologically stressed persons with association of fatigue significantly lowered cortisol response and increased mental performance compared to placebo [125]. Salidroside present in *R. rosea* exerts its antistress, adaptogenic, and antidepressive activity through stimulation of neuropeptide-Y with concomitant lower levels of HSP-72 (heat shock protein) release in brain microglial cells, ultimately modulating the HPA axis stress response [126].

3.6 *Lavandula angustifolia*

This is also known as lavender, containing aromatic oils that mainly contain triterpenoids such as linalool. Its aromatic effect is reported to display

antistress and mood relaxing activities by biological effects on both stressed animal and humans. In a trial among 24 human subjects experiencing experimental stress, linalool inhalation resulted in a decrease in BP, HR variation, and salivary cortisol levels, thus modulating the stress response [127]. Simultaneously, other studies have also claimed the cortisol and daytime BP-lowering properties of lavender oil aroma in 83 stressed prehypertensive subjects [128]. Lavender oil inhalation in another study among students performing arithmetic tasks as a mental stressor gave a stress-relieving action in aspects of symptoms and lowering of stress biomarkers cortisol and chromogenin levels in saliva [129]. A randomized trial also confirmed the stress-relieving property of lavender oil among female patients suffering from urinary incontinence [130]. A further study of healthy volunteers also clarified that after sniffing lavender oil aroma, significant reductions of stress hormone cortisol are achieved [131]. Linalool's effect upon modulation of hypothalamic gene expression in stressed rats may be one of the pathways to preventing elevation of cortisol levels during stress [132,133].

3.7 *Bacopa monnieri*

Commonly known as brahmi, *B. monnieri* is a widely explored brain tonic with huge amounts of existing scientific evidence in the area of cognitive treatment. Chronic administration of *B. monnieri* in rats ameliorates forced swimming-induced hypothermia, which is a parameter of stress evaluation in rodents [134]; also chronic immobilized stress-related pathological changes upon spleen, adrenal gland, and metabolic enzyme activities in mice are significantly prevented by chronic *B. monnieri* treatment [135]. A study has indicated that chronic stress-induced increase in brain HSP-70 levels, specifically in the hippocampal area, is only prevented by chronic pretreatment with *B. monnieri*, with a simultaneous increase in superoxide dismutase activities in the cortex area [136].

A clinical study with *B. monnieri* has been conducted on human volunteers to validate antistress properties. A double-blind, crossover, placebo-controlled study demonstrated that upon consumption of *B. monnieri* extract (320, 640 mg), volunteers showed a significant positive level of mood with a significant lowering of cortisol levels after postmultitasking experimental mental stressors. In contrast, the placebo group showed a higher cortisol level and negative mood at poststressors [137].

3.8 *Ginkgo biloba*

Animal findings suggest that the flavonoids and terpenoids present in this ancient Chinese herb improve stress adaptation to animals through normalization of alterations in brain catecholamines and plasma cortisol levels [138,139]. The versatile cerebroprotective role of this herb led to research that

showed that chronic stress-induced detrimental changes in discrimination learning, cognitive functions, and plasma stress hormones in rats are suppressed by chronic treatment of *G. biloba* (GB) extracts, resulting in facilitation of better behavioral adaptation under stressful situations [140,141].

Standardized extract of GB consumption in a clinical trial upon healthy young volunteers under experimental mental stress has also demonstrated significant lowering of stress-induced BP and salivary cortisol levels, without any effect on HR [142]. Ginkgolide, a bioactive component, demonstrates an effect upon adrenal gene expressions of glucocorticoids, which also causes lowering of circulating cortisol levels in stressful situations in animals [143].

3.9 *Ocimum sanctum*

This is commonly known as holy basil, and one of its isolated phytoconstituents, ocimumosides A from leaves, displays a promising antistress action on rats under stress, with normalization of stress-induced elevation of cortisol, glucose levels, and adrenal hypertrophy [144]. Also chronic restraint stress-induced anxiety and depression in mice is well reversed by oral *O. sanctum* treatment [145,146], with reports of efficacy in swimming models of stress exposure [147].

In a controlled clinical trial among patients suffering from stress-related anxiety disorder, *O. sanctum* capsules of a high dose of 1000 mg per day for 60 days were administered with a control placebo, because a high dose of *O. sanctum* has shown significant antistress effects in a rodent swimming model of stress. The study concluded that *O. sanctum* treatment significantly improved behavioral aspects of psychological stress as evaluated by a stress questionnaire [148]. *Ocimum tenuiflorum* is another species of *O. sanctum* and has also been further clinically tested in 150 psychologically stressed patients in a randomized double-blind trial. The outcomes of the study after 6 weeks depicted that stress-related symptoms such as forgetfulness, exhaustion, and sexual problems of recent origin were significantly improved compared to placebo [149].

3.10 Black Tea

Black tea is enriched with theaflavin, which has good antioxidant properties. Besides this, theaflavin-enriched black tea extract has also shown antistress effects in a number of randomized crossover studies. In a study a group of college students was given black tea extract for 9 days and were gone through stressor, and subsequently serum cortisol levels were significantly lower compared to placebo treatment [150]. Black tea polyphenols exert an antistress and antidepressant action against chronic stressors in mice through a direct alteration of brain monoaminergic responses and antioxidant status [151]. Another parallel group study among 75 healthy nonsmoking men revealed that

6 weeks of continuous black tea consumption led to significant lowering of psychological stress together with lower cortisol levels and reduced platelet activation upon challenge of mental stressors. Moreover, in that study the placebo group received a caffeinated dummy that showed insignificant effect on stress reduction, thus suggesting the role of black tea constituents other than caffeine [152]. Thus apart from the CNS stimulating effects of caffeine from a cup of tea, stress and fatigue-alleviating properties are related to the content of other natural substances present in it.

3.11 Green Tea

Green tea polyphenols prevent cognitive dysfunctions associated with psychological stress [153]. Green tea is fully enriched with the amino acid L-theanine, which is also reported to reduce mental stress levels in humans. L-Theanine consumption in high-stress-response human adults efficiently reduces anxiety and BP elevation specifically after acute psychological stressors [154]. In a randomized crossover design study among students, intake of green tea was responsible for improving stress scores and mood profile, with a decrease in mental stress load-induced salivary chromogranin A levels [155]. A double-blind study concluded that L-theanine consumption in healthy subjects led to reduced HR variation and salivary immunoglobulin-A levels when subjected to a psychological arithmetic task stressor [156].

Catechin, a major constituent like tannins in green tea, is reported to ameliorate corticosterone injection-mediated stressful behavioral alteration in rats through modulation of the HPA axis [157]. Green tea's antistress assessment might be correlated with catechin's, especially epigallocatechin gallate found in green tea, which is a potent inhibitor of the cortisol-producing enzyme 11β-hydroxysteroid dehydrogenase type 1, leading to lowering of cortisol levels [158] (Fig. 14.2).

4. NUTRITIONAL THERAPY

4.1 Vitamin C

Vitamin C or ascorbic acid is abundantly found in various citrus fruits, and has an antistress effect by lowering cortisol levels in stressful situations. Reports indicate that in a double-blind study among 60 human subjects, continuous 2-week high vitamin C consumption prevented psychological stress-induced systolic BP and cortisol elevation in the stressful situations of public speaking and mental arithmetic tests [159]. In clinical studies in marathon athletes supplemented with vitamin C, postracing serum cortisol level elevation was reported to be significantly lower than placebo-treated athletes, which reflects the stress-reducing action of vitamin C during severe stressors [160]. Thus a regular intake of a diet enriched with vitamin C could be a simple strategy for controlling stress cortisol levels.
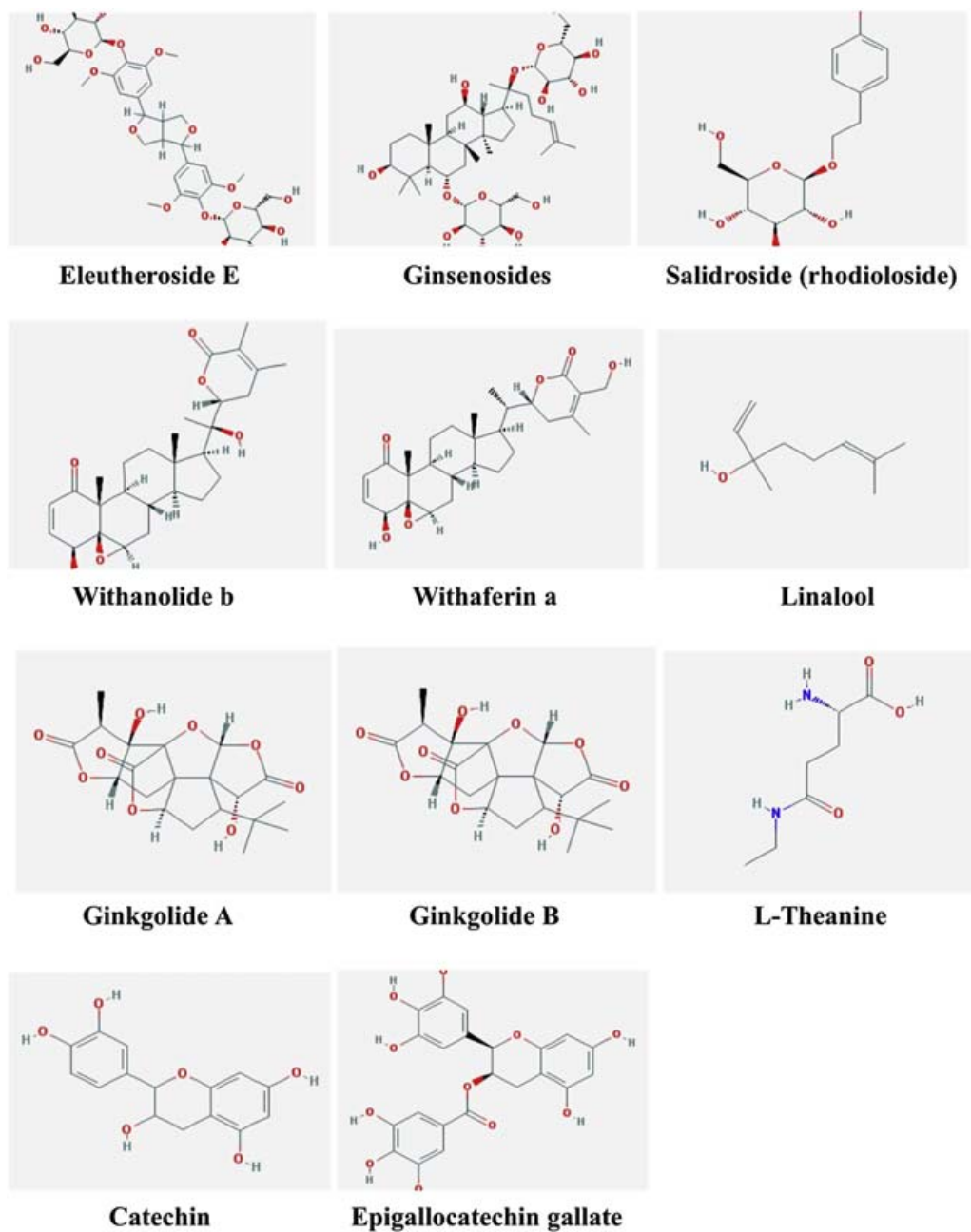
FIGURE 14.2   Respective lead molecules responsible for activity.

4.2 L-Lysine

This is an essential amino acid abundantly found in foods such as spirulina, fenugreek seeds, fish, eggs, meat, etc. The result of a double-blind, placebo-controlled study on 180 adults facing psychological stress confirmed the antistress effect of the amino acid through significant lowering of anxiety and cortisol levels [161]. A 3-month, randomized, double-blind study among a

poor Syrian community validated that lysine-fortified wheat consumption resulted in significantly reduced chronic anxiety and lower plasma cortisol response to stress [162].

4.3 L-Ornithine

L-Ornithine is a nonessential amino acid usually contained in protein-rich foods such as fish, meat, eggs, and also nuts. A randomized placebo-controlled trial suggested that 400 mg of L-ornithine consumption in adults led to a marked decrease in negative feelings, stress score, and salivary cortisol levels with a better quality of sleep [163]. In a placebo-controlled trial among 52 women having high levels of mental stress, L-ornithine intake for 8 weeks resulted in a marked improvement in sleep quality and mood states, and also a decrease in serum cortisol levels and perceived mental stress [164].

4.4 Jerte Valley Cherries

These cherries are specially grown in Spain and contain high levels of tryptophan, serotonin, and melatonin. One study claimed that consumption of these cherries in young, middle-aged, and elderly subjects controlled anxiety and perceived stress levels with a significant lowering of urinary cortisol levels and increased serotonergic activity, suggesting that regular consumption of these cherries may reduce stress levels [165].

4.5 Fish Oil

Fish oils (FO) are reported to contain larger amounts of polyunsaturated fatty acids such as omega-3 fatty acids, eicosapentaenoic acid, docosahexaenoic acid, etc., which are widely accepted as beneficial for human health. In alcohol addicts going through rehabilitation programs, 3 weeks of FO supplementation provided a significant reduction in anxiety-distress symptoms and cortisol levels as compared to before treatment and placebo subjects [166]. In another study, reduction of plasma epinephrine, cortisol, and psychological stress response was significant in volunteers taking FO when subjected to experimental mental stress [167]. The stress-related cortisol-lowering action of 6 weeks' FO consumption was also evident in another trial among healthy adults [168].

4.6 Soy Protein

Soy protein is a herbal protein that contains mixtures of lecithin phosphatidylserine and lecithin phosphatidic acid. Several clinical trials with the isolated phosphatidylserine-phosphatidic acid (PAS) mixtures from soy protein had confirmed a protective effect on mood and HRs against severe stress.

Another trial among 75 chronically stressed patients showed that PAS consumption for 42 days effectively reduced the stress-associated increase in serum ACTH and cortisol levels [169]. More trial data also cite that consumption of isolated soy protein complex or PAS in humans gave greater protection against psychological stress and a pronounced decreasing effect on serum ACTH and cortisol concentrations in respect to placebo treatment [170]. Decreased HPA axis-mediated elevation of serum cortisol after stressors was also further evident from another human clinical evaluation with phosphatidylserine consumption [171].

4.7 Casein Tryptic Hydrolysate

Casein is the major milk protein that is hydrolyzed by both pepsin in the stomach and trypsin in the intestines of adults, whereas in infants it is only hydrolyzed by trypsin due to inactive pepsin in a neutral gastric environment. Research has found that the reason behind the calm state of infants after having milk is due to the formation of tryptic hydrolysate of casein in the intestines resulting in the generation of a specific peptide, after cleavage at 91 to 100 number amino acid chain, called α-casozepine, which gives a relaxing effect [172]. In animal models of stressful anxious behavior, the bioactive peptide α-casozepine-enriched casein hydrolysate showed potent activity when compared to benzodiazepines without any side effects such as tolerance and sedation, as seen with diazepam [173,174].

In a randomized, double-blind, placebo-controlled trial among 42 healthy volunteers, two capsules of casein tryptic hydrolysate (CTH), 200 mg three times at 12 h intervals, resulted in better recovery from mental and physical stress. Major findings in that study were that CTH significantly prevented the rise in systolic BP and plasma cortisol levels during the mental stress test [175]. In another crossover, double-blind, placebo-controlled trial among 63 women, CTH 150 mg per day ingested for 30 days significantly reduced the severity of stress-related symptoms. Specifically among 44 stress symptoms, greater improvement was reported in digestive, cardiovascular, intellectual, and emotional aspects of stress-associated manifestations [176].

4.8 Yoghurt

In a double-blind study, consumption of yoghurt enriched with α-lactalbumin, casein tripeptides had been shown to increase the capability to cope with stressful situations. The study pointed out yoghurt's beneficial effect upon psychological stress challenges and resulted in better HR recovery, decreased anxiety, and increased positive mood with lower salivary cortisol levels [177].

4.9 Whey Protein

α-Lactalbumin is a bovine milk-derived protein, also called whey protein. In a randomized human trial it was quite evident that lactalbumin or whey protein consumption in healthy adults increased their coping ability with stressful situations, with a lowering of cortisol levels [178]. Another placebo-controlled human trial also confirmed the stress-relieving effect of whey protein consumption by significantly lowering cortisol levels after stressful exertion [179].

Herbs	Antistress activities
Polygala tenuifolia	Ameliorated chronic mild stress (CMS) and reward insensitivity in rats by reducing serum cortisol, ACTH and CRH levels [180]. Significantly increased sucrose intake and decreased cortisol elevation in chronic mild stress (CMS)-treated rats [181]. Inhibited the decrease in brain derived neurotrophic factor due to CMS [182].
Schisandra chinensis	Treated rats after stressful swimming exercise showed lower values of blood glucose, cortisol, IL-1 and IL-2 levels compared to placebo ones [183]. In isolated neuroglia cells, it modulated in vitro expression of neuropeptide-Y (NPY) and heat shock protein (HSP), the molecular mediators in tolerance and adaptation of stress response [184].
Argyreia speciosa	Significantly lowered the swimming and chronic immobilization stress-induced elevation in cortisol and adrenal gland hypertrophy in treated rats [185]. Pretreatment in rats subjected to cold restraint stress prevented adrenal gland hypertrophy and showed stress ulcer protective and cortisol-lowering properties [186].
Andrographis paniculata	Attenuated the elevation of cortisol, TNF-α, IL-10 and prevented chronic stress-induced pathological changes in rats subjected to chronic unavoidable foot shocks [187]. Also been reported to alter the behavioral pattern, affecting spontaneous motility in rats [188].
Pomegranate peel	In CMS rats treated with extract it showed lower serum cortisol concentrations with an increase in sucrose intake compared to placebo [189]. In rats fed with high-fat diet, extract significantly decreased glucose intolerance, dyslipidemia, TNF-α, IL-6 and cortisol levels compared to control group as metabolic syndrome indicator [190].
Mellisa officinalis	In chronic stressed mice it prevented the physical changes of spleen, thymus, and body weight with a lowered serum cortisol concentration compared to control [191]. Increased neuroblast differentiation and lowered serum cortisol, GABA transaminase levels [192].

Antistress activity of some lead plant extracts and fractions in animal models pending future human trials.

5. CONCLUSION

There is a potential to consider psychological stress management therapeutically by natural substances, which is the safest way of shielding progression to chronic stress-related diseases. Validations exist for pathophysiological connection between stress and various disorders, as proven by globally conducted numerous cohort studies and metaanalyses. As discussed through literature surveys, numerous clinically proven herbal or natural remedies for stress-related disruption of the HPA axis could be advised for increasing patients' adaptability to altered homeostasis for positive outcomes. Of these herbs and dietary interventions, many are effectively confirmed to be devoid of any side effects, with a modulating action on HPA axis-mediated stress hormone levels status; however, a larger amount of clinical data are still awaited for its universal acceptance in specific stress-related diseases. Many of the plant-based herbal drugs and nutritional substances discussed in this chapter have been marketed as antistress, with exclusive patent rights in various countries under dietary supplements or neutraceuticals. Also the active phytomolecules responsible for their antistress activity, as mentioned in Fig. 14.2, could reflect the need for standardization of natural products to be efficacious. Although in future, more randomized controlled trials in large number of subjects with a strong pharmacovigilance-oriented monitoring are required for a FDA approval & recognition. Thus a robust approach must be emphasized for recognition and acceptance by modern prescribing clinicians [193], due to US Food and Drug Administration (FDA)'s and other stringent regulatory authorities' recognition for their acceptance.

Also from a clinician's and prescriber's point of view, more rigorous clinical trials with standardization of these herbal and natural formulations need to be undertaken to validate specific antistress therapies for USFDA approval or be categorizing as antistress nutraceuticals or dietary ingredients, which remains to be resolved. However, traditional usage of natural plant substances and foods with validation through modern studies could be considered for their consumption. Thus along with the prime medications intended to treat various chronic diseases, novel nutritional interventions with plant-based therapies could be considered for effective and safer antistress therapy along with other chronic disease management to be completely beneficial for patients. Besides very commonly and routinely used standard low-dose SSRI antidepressants, anxiolytics, etc., this novel dietary and herbal mode of therapy could replace them and justify their usage for specific modulatory action upon stress biomarkers. We also hereby emphasize the urgent need for antistress herbal drug discovery research programs globally, to design and deliver potential candidates in the future for modulating the human body's ability to adapt to stressors.

ACKNOWLEDGMENTS

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National Seminar on

RECENT ADVANCES AND SCOPE IN HERBAL TECHNOLOGY : CHALLENGES AND PROSPECTS

RASHTCP-2016

Organized by

DEPARTMENT OF PHARMACEUTICAL SCIENCES
ASSAM UNIVERSITY, SILCHAR, INDIA
9th & 10th September, 2016

CERTIFICATE OF RECOGNITION

This is to certify that _Mr. Dhrubojyoti Mukherjee_ of _Dept. of Pharm Tech. Jadavpur University_ has participated in the National Seminar on 'Recent Advances and Scope in Herbal Technology' organized by Department of Pharmaceutical Sciences, Assam University, Silchar, India held during September 9th and 10th 2016 and delivered the Lecture/Presented the paper(oral/~~poster~~) entitled '_Total steroid and Terpenoid enriched fraction ...... nitric oxide Synthase expression_' as invited ~~Speaker~~/Participant /~~Delegate~~ / ~~Scientific Presenter~~ / ~~Chairperson~~.

Dated, Silchar
10th September 2016

Prof.(Dr.) Sanjib Das
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Gelsemium Sempervirens L. Improve Memory And Cognitive Impairment In Mice Scopolamine...

........................**Oral Presentation**..

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on the occasion of National Conference on 'Intellectual Property Rights of Pharmaceuticals 2.0'
held on Saturday, May 7, 2022 at PSIT, Kanpur

Dr. Ashish Srivastava
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